Chalones: Concepts and Current Researches

MONOGRAPH 38

NATIONAL CANCER INSTITUTE MONOGRAPH 38

JULY 1973

Chalones: Concepts and Current Researches

DHEW Publication No. (NIH) 73-425

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NATIONAL INSTITUTES OF HEALTH
NATIONAL CANCER INSTITUTE, BETHESDA, MARYLAND 20014

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CHALONES: CONCEPTS AND CURRENT RESEARCHES

Proceedings of the
First Symposium of the
International Chalone Conference
Held at
Brook Lodge, Augusta, Michigan
June 5–7, 1972

Conference Committee:
John C. Houck, Ph. D.
Edna Laurence, Ph. D.
Henry Hennings, Ph. D.
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Miscellaneous Chalones

Over the last decade, an increasing number of workers have been describing results consistent with the concept of the "chalone," or specific endogenous mitotic inhibitor. There now are reports in the literature describing or suggesting the existence of at least 10 different chalones, with obviously more to come. Because of the enormous intrinsic importance of the idea of the chalone to both biology and clinical medicine and because of the evolution of the state of art of isolation and purification procedures for proteins and glycoproteins over the last few years, it was decided to convene an international conference of the primary workers in the area of chalones. Support for this was obtained through the kind auspices of the National Cancer Institute and the Office of Naval Research. Both of these federal agencies felt that, since their mission was involved in chalone research and since the great bulk of the chalone work (as well as the concept itself) was essentially developed overseas, they should provide funds to support a domestic conference on this subject. The Upjohn Company was kind enough to provide the facilities of their beautiful Brook Lodge.

The organizing committee then attempted to cover the world literature and invite to this conference almost every significant investigator of chalones over the past decade. In addition, the organizing committee invited a small and very select number of critical cell biologists interested in and capable of evaluating the chalone concept, even though the concept was not directly involved in their own work. In addition to this audience, a select number of senior administrators who had responsibility for research in private foundations, industry, and the governments of both the United Kingdom and the United States were invited. Finally, it was felt that, because this was a closed meeting, the proceedings of this conference would reach its most important and largest audience via publication as a monograph by the *Journal of the National Cancer Institute.*

The activities at this conference, which was essentially an intensive 3-day exposure to chalones, their evidence, and the criticism of their evidence for existence, were extensive and wide-ranging. Attempts were initially made to include the discussion in these proceedings. This proved to be almost impossible because of both space limitations and the enormous difficulties involved in editing, as well as the prohibitive amount of time involved which would contribute to a significant delay in publication. Certain select discussors were kind enough to provide us with copies of their remarks, which are included following the appropriate paper.

"I should have mentioned that we invited a few devil's advocates."

It was felt by all the participants in this conference that chalones are important as the most probable mechanism of negative-feedback inhibition of mitosis. It was also felt that, because of the importance of their potential to clinical medicine and to fundamental biology, a much larger number of scientists should focus their attention on the chalone concept and be urged to test this concept experimentally. The organiz-

ing committee hopes that the proceedings of this conference will prove sufficiently provocative and evocative to this end so that a larger number of workers around the world will consider the chal-

one concept and explore rigorously its relevance and reality for the particular systems they are interested in.

John C. Houck
Edna B. Laurence
Henry Hennings
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Organizing Committee
International Chalone Conference
October 30, 1972

General Introduction to the Chalone Concept [1]

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IN THE mid-1950's, Paul Weiss of New York and Olav Iversen of Oslo separately began to apply the then very current concepts of cybernetics to biology; in particular, they applied the concept of the negative-feedback inhibitor to the control of mitosis by normal cells. This theoretical concept was applied by Bullough and Laurence of London to their experimental data relating to the control of the mitosis of epidermal cells in vivo and in organ culture. These latter workers developed the concept of a specific and endogenous mitotic inhibitor which, for reasons described elsewhere in these proceedings, they decided to call a "chalone" [kay-lone].

The essence of the chalone concept is that it represents a class of compounds, as yet to be fully defined chemically, which are capable of *specifically* inhibiting the mitotic activity of a given cell type. This cell specificity is *not* associated with any species specificity, and hence the practical problem of obtaining chalones for experimental work can be resolved by using extracts of the appropriate animal organ. A most important characteristic of the chalone is that it is not cytotoxic; the original concept of Bullough and Laurence indicated that this mitotic inhibition was essentially reversible. This reversibility would suggest a significant lack of cytotoxicity. The simplicity and elegance of the idea of a specific endogenous mitotic inhibitor, or chalone, controlling the mitosis of normal cells are very compelling and very exciting. It is also amenable to experimental test. As the subsequent chapters of these proceedings will indicate, a number of workers have attempted to design rigorous experimental tests for the existence and nature of chalone control of the mitosis of a wide variety of cells.

The difficulty in implementing and developing experimentally the chalone concept is that it requires a fairly unique marriage of sophisticated cell biology and sophisticated protein chemistry. Thus, the primary criticism of chalones in the past has been the fact that no one has been able to isolate and purify these materials to a degree convincing to biochemists. Conversely, biochemists capable of applying the recently developed arts of isolation and purification of chalones have not been using biological systems that are convincing to sophisticated biologists. One of the major purposes of these proceedings is not only to report on the existence of chalones from various systems but also to indicate, insofar as possible, the primary problems and difficulties in experimentally delineating the chalone. It is hoped that, by developing a shared vocabulary between cell biologists and biochemists around the issue of chalones, an enormously important interfacial area of scientific development could be more successfully dealt with. As is usual with modern biomedicine, the interdisciplinary areas are both the most difficult and the most exciting to come to grips with.

The primary technical problems with establishing a chalone experimentally revolve around two aspects: assay technique and artifacts, and cytotoxicity.

Essentially there are three ways of measuring the mitosis of a cell: 1) to count mitotic figures

[1] Presented at the First Symposium of the International Chalone Conference, Brook Lodge, Augusta, Mich., June 5–7, 1972.

which have been trapped in metaphase by colcimid or by vinblastine, 2) to measure the uptake by cells of tritiated thymidine as a measure of their entrance into "S" phase, and 3) to actually count the number of cells in an attempt to determine how many more there are after a given period of time.

Experimentally, the most inadequate technique is the incorporation of ^{3}H-labeled thymidine. There are at least two major reasons why this technique must be used with considerable care. First, a number of tissues contain enzymes such as thymidine kinase which can alter the labeled thymidine prior to its incorporation into a cell. Thus, in this fashion, an apparent mitotic inhibition could be produced by an extract of tissues which contains this thymidine kinase. It is important to remember that both the reticuloendothelial system and the liver contain very significant amounts of this enzyme. In part, this objection can be overcome by appropriate controls as well as by the direct determination of thymidine kinase activity. It is interesting to note that extensive dialysis, against water, of extracts containing thymidine kinase usually results in the loss of enzyme activity in the supernatants of these extracts. A second problem, relating particularly to small-molecular-weight chalones, of which there seems to be evidence for at least three (liver, melanocyte, and granulocyte), is contamination of the extract with unlabeled or "cold" nucleosides and nucleotides, including thymidine. These small-molecular-weight nucleosides and nucleotides will dilute out the "pool size" of the labeled thymidine being added to the system under study. Thus, an apparent inhibition of uptake of thymidine and hence of entrance of cells into "S" phase could be a result of simply decreasing the probability of the cell incorporating a specifically labeled thymidine molecule. Again, this "pool size" dilution problem, which is not unique to chalone studies, can in part be obviated by extensive dialysis of the tissue extracts to remove the small-molecular-weight nucleosides and nucleotides. This latter technique, however, is not appropriate to studies of tissue extracts containing dialyzable small-molecular-weight chalones.

The question of cytotoxicity of tissue extracts is a far more difficult criticism of the experimental evidence for the existence of chalones. We know that the extracts of a wide variety of tissues contain a wide variety of cytotoxic materials. Probably the most important quantitatively is complement. The latter portions of this chain of interdigitated enzymes which constitute the complement system contain a phospholipase and other hydrolases which are, by definition, cytolytic. Thus, any portion of the complement system which might be contaminating the tissue extract and which might be activated during the process of partial purification would lead to the destruction of cells and therefore an apparent inhibition of mitosis. This leads to a fundamental discovery, i.e., Houck's Law: "Dead cells do not divide." An important corollary to Houck's Law is, "Dying cells divide damn slowly."

Houck's Law, then, simplistically states the primary criticism of any work using tissue extracts to demonstrate an inhibition of mitosis of any given cell type. It is impossible to prove that a cell is not being cytotoxically rendered incapable of normal mitotic rates. Historically, the evidence for cytotoxicity is usually based on the ability of cells to exclude a vital dye. However, this may be a very late manifestation of a response of the cell to toxic materials. We have attempted to explore the kinetics of cell death in terms of what happens to the various marker systems used to distinguish viable from nonviable cells. Fibroblasts which have been subjected to a lethal dose of radiation will very quickly lose their ability to incorporate tritiated thymidine. Some hours later they start to lose the ability to incorporate labeled uridine and then labeled phenylalanine. A number of hours will then pass without any significant alteration in the other metabolic markers of viable cells. Then, fairly quickly in terms of kinetics, the cell starts to release previously incorporated radioactive chromium and to discharge various lysosomal enzymes. Shortly thereafter, the cytoplasmic enzyme, lactic dehydrogenase, starts to be released from the cell into the extracellular space and the cell will no longer exclude vital dyes. Subsequently, even as the morphology of the cell is being destroyed, one can still detect statistically significant amounts of phosphorylation,

oxygen consumption, and glucose utilization. The lifetime of the mitochondrion is considerably longer than that of the rest of the cell, and this organelle will remain viable for many hours past the obvious death of the host cell. The catabolism of cytoplasmic enzymes such as those involved in glycolysis may take some time, during which the cell apparently can still consume glucose.

Thus, the question of when death occurs for a cell is as complicated as when death occurs for an organ or even a human being. Consequently, it is possible that a marginally cytotoxic agent could be applied to a given cell type and, by virtue of making the cell sufficiently "sick," could inhibit the enormous energetic expenditures involved in entering "S" phase; yet, during this period, it might not significantly alter any of the various markers used to distinguish viable from nonviable cells. The reversibility of this phenomenon would simply mean that the cytotoxic agent was not sufficiently powerful or was not in sufficient concentration to effect irreversible changes. Yet, in fact, it was very slowly killing the cell and thereby inhibiting mitosis!

One possible argument about the existence of a cytotoxic agent would be that the effect of the chalone is known to be specific for a given cell type, while presumably a cytotoxic agent would not be. This assumption may not, in fact, be true. It is known that certain kinds of cells—e.g., lymphocytes—in the circulation have a different chemistry about their surface membranes than do other cells—say, fibroblasts, which grow attached to glass. It might well be that the fibroblast attached to glass would not be affected by a mildly cytotoxic agent, whereas the lymphocyte would be. This could occur either because part of the fibroblast is masked, being adsorbed to the glass on which it is growing, whereas all of the lymphocyte is equally exposed to attack, or it might also be due to intrinsic differences in the susceptibility or the number of binding sites on the appropriate cell for the mildly cytotoxic agent.

Perhaps the best technique for the elimination of primary cytotoxic events is to study the process in vivo. One has to assume that the liver, kidney, and other methods of detoxification of various cytotoxic materials, including dilution, would limit very markedly the amount of cytotoxic material that could be administered and which would be apparently inhibiting the in vivo biological system under study. Needless to say, the complications of in vivo studies of chalones are both historic and obvious. Therefore, it is enormously difficult to exclude rigorously the possibility of cytotoxicity complicating the interpretation of data demonstrating the inhibition of the various indices of mitosis that are available. Hence, the best one can do to render cytotoxicity improbable would be to demonstrate that the chalone action seems to be cell specific and reversible, and that the inhibition of uptake of tritiated thymidine was not associated with any inhibition of the uptake of radioactive amino acid (such as phenylalanine) or of uridine.

Our evidence suggests that both fibroblast and lymphocyte chalones are located on or near the surface of cells. Each cell has a particular kind of signal which alters or displaces the chalone so that the cell then can enter "S" phase. Our preliminary data, which are still inadequate, suggest that the most likely mechanism for the action of chalones is that the chalone in some way obscures or prohibits the entrance of calcium into the cell. Diploid lymphocytes and fibroblasts stringently require calcium for mitosis, and the proportion of cells entering "S" phase or dividing is very much contingent upon the presence of calcium. This calcium requirement is not demonstrable for heteroploid lymphocytes or heteroploid fibroblasts in culture, however. Once calcium enters the cell, it can stimulate the cyclic AMPase activity of one of the two major 1,2-phosphodiasterases known to exist in most cells. One of these enzymes with a fairly high K_m will, in the presence of calcium, demonstrate a fourfold to tenfold decrease in K_m and hence a remarkable increase in activity. Perhaps this is analogous to the magnesium- or manganese-dependent activity of various types of ATPases. At any rate, as a result of the entrance of calcium from the medium into the cell, the activity of this diasterase is markedly increased, and a considerable amount of cyclic AMP in the cell is hydrolyzed. For unknown reasons, hydrolysis of cyclic AMP in turn seems to stimulate the en-

trance of the cell into "S" phase. This is to some extent discussed in the paper by Smulson. Needless to say, this proposed mechanism of the action of chalone—exclusion of calcium from the cell under normal circumstances—remains to be established definitively.

Inevitably, during the course of discussion of chalones, there arises the question of the utility of the concept in terms of clinical medicine. We believe that there are at least two major areas of application to human disease. First, and primarily, the application of the lymphocyte chalone to normal lymphocytes in terms of immunosuppression could be extremely important. Data described by us in a paper in this collection indicate that the heteroploid or leukemic lymphocytes do not bind chalone very well, and three to four times as much chalone must be used to inhibit the mitosis of these cells as would be required to inhibit the transformation of normal lymphocytes. This inability of tumor cells to bind chalone as effectively as the normal parent cells is also seen in the case of the simian virus 40-transformed human fibroblast. Thus, it would seem most logical that the most effective inhibition of mitotic activity of cells would be in the normal diploid cell rather than the abnormal heteroploid cancer cell.

Our evidence suggests that lymphocyte chalone can effectively inhibit the transformation of normal human lymphocytes, whether they are stimulated by mitogens or antigens. The ability of lymphocyte chalone concentrates to inhibit specifically mixed lymphocyte transformation suggests very strongly that the possibility of immunosuppression by lymphocyte chalone is a very real one. The data discussed by Kiger and Florentine, by Garcia-Gerault, and by Chung indicate that this type of immunosuppression can be, in fact, demonstrated in vivo.

The second and most obvious application of chalones of all kinds would be in terms of control of cancer. It seems to me quite a remote possibility that one could administer for a long enough period large enough amounts of chalone to patients to alter their clinical course significantly in terms of tumor growth. However, it might very well be possible to administer for short periods of time sufficiently large amounts of chalone to inhibit both normal and abnormal cells, during which all the cells that would have been in "S" phase or G_2 will have completed their mitotic cycle and re-entered G_1. At this time, a significant reduction in the circulating concentration of chalone would permit the tumor cell to divide in a synchronized manner, while the normal cells would still be suppressed mitotically. The ability to synchronize tumor cells would be specific only for this cell type in the whole organism and would then render these cells considerably more vulnerable to attack by chemotherapeutic antimitotic agents. In this fashion, by developing synchrony in the tumor cell and protecting by mitotic suppression the normal cell, it may be possible to increase to an enormous degree the efficacy of the various existing techniques of chemotherapy and radiation therapy in tumors. This would, in fact, be an extremely important advance in chemotherapeutic techniques and alone would justify the scientific and financial investments in chalone research.

The Chalones: A Review [1]

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THIS IS a brief review, partly historical, of the studies that have been made on the problem of cell replacement in adult mammalian tissues and on the role played in this process by tissue-specific chalones. It also naturally encompasses the manner in which the controlling mechanism is broken when tumor growth begins.

In the past this interrelated group of problems has often been considered solely in terms of the mitotic rate, since it is obvious, as in hyperplasia and in hormone-stimulated tissues, that an increased rate of cell gain commonly accompanies an increased tissue mass. Only relatively recently has it been appreciated that cell loss, which is the other side of the equation, is not normally accidental or determined by wear and tear but is as positive a process as is cell gain. The rate of cell loss is a function of the rate at which the postmitotic cells age, and it is now known that the rate of aging is controlled by a part of the same mechanism that also controls the mitotic rate. In fact the rates of these two processes vary in apparently perfect unison and are determined from moment to moment by the intracellular conditions [*see* (*1,2*)].

Also involved is the question of tissue function. It has long been appreciated that mitotic activity and tissue function are mutually exclusive cellular activities. However, tissue function is characteristic of postmitotic cells, and it is now becoming clear that the real alternative cellular activities are mitotic activity and postmitotic cell aging; tissue function, although normally associated with cell aging, is pathologically separable from it (*1,2*). It now also appears that the choice between the alternative cellular activities is made in terms of the activation of "mitotic genes," which specify the syntheses necessary to the mitotic cycle, or of "aging genes," which direct the process or the speed of the process by which a cell ages and passes to its death (*3*).

These are the conclusions of the moment. What follows is an introduction to the evidence on which they are based.

METHODS OF CELL AND TISSUE REPLACEMENT

Each adult mammalian tissue must possess a potential that is adequate to meet all the metabolic demands that are likely to be put upon it. Basically this is a question of tissue mass, which is itself a function both of cell number and of cell size. It has often been suggested that an increased metabolic demand may cause a tissue to increase its cell number, but firm evidence for this is still lacking. Rather, it seems that cell number is genetically determined at a value that is adequate to meet the probable maximum metabolic demand. However, since, in most tissues, cells are constantly being gained and lost, what is actually genetically determined is the mechanism that fixes and maintains the cell number.

It is a common observation that the mass of any tissue tends to remain remarkably constant and also that, when tissue is lost by damage, the normal mass—no more and no less—is quickly regenerated. This second point shows clearly that the control mechanism does not operate at a constant level; it reacts quickly to the needs and circumstances of the moment.

In some tissues the degree of activity of this

[1] Presented at the First Symposium of the International Chalone Conference, Brook Lodge, Augusta, Mich., June 5–7, 1972.

control mechanism may also be varied by normal physiological factors. Thus, the mitotic activity of the hair roots varies widely according to the state of the hair growth cycle, in a manner not yet understood. Evidently, within each hair root there exists some alternating factor, possibly genetic in origin, which interferes with the normal control mechanism to produce the observed rhythm (*3*).

Another complexity is seen in hormone-dependent tissues. In the presence of the appropriate hormone the mitotic activity of the target tissue increases greatly and so too does the tissue mass. The indication is that the hormone partially neutralizes the control mechanism and so allows a form of overgrowth that in all its particulars is closely similar to hyperplasia (*2,3*).

In the few tissues that are nonmitotic, notably the skeletal and cardiac muscles and the neurons, the cell number is static except for a low rate of cell death (*3,4*). However, this cell number is established in the normal way during fetal and early life when these tissues are still mitotic. Later tissue growth is by increasing cell size. In the muscles, this type of cellular hypertrophy can occur at any time in response to an extra metabolic demand (*5*). Nonmitotic tissues are characterized by a constant replacement of cytoplasm, and this may also occur with lesser intensity in the cells of mitotic tissues.

These all are examples of the renewal of tissues by their own cellular activities, whether by mitosis or by cytoplasmic synthesis. There is, however, another method of tissue renewal which depends on the continuing existence of a relatively undifferentiated stem cell population. Thus, both granulocytes and erythrocytes continue to be formed by terminal embryonic type differentiations from a population of pluripotential stem cells in the bone marrow; plasma cells continue to be formed from a stem cell population in the lymphoid system (*3*); and there is reason to believe that the various connective tissues may also be dependent on a common stem cell population which can move throughout the body in the bloodstream [*see* (*6*)]. In such systems, each terminal differentiation evidently depends on the action of a particular inducing substance and, as is the rule in the embryo, once taken, this step is irreversible. In all cases the new tissue cells formed in this way then undergo mitotic activity which is controlled and finally stopped by the usual tissue control mechanism.

OLDER EVIDENCE

The study of tissue control mechanisms may be said to have begun with experimental analyses of wound healing, notably in the skin, and of organ regeneration, notably after the partial ablation of kidney or liver [*see* (*3,7*)]. Regarding wound healing, there are three successive phases: 1) cell migration, by which the gap is closed but about which little except descriptive accounts exist even today; 2) cell division, by which the lost cells are replaced; and 3) cell maturation, through which the newly produced cells become functional. Originally, in the hormone-conscious fashion of the time, it was thought that this whole regenerative response must be induced by a "wound hormone" secreted by the dead or injured cells. This theory, unsupported by any hard fact, had a remarkably long life of more than 50 years. Regarding the phase of cell division, it was finally destroyed by Bullough and Laurence [(*8*); and *see* (*9*)] who showed that the mitotic stimulus is in fact due to the local loss of a previously present mitotic inhibitor.

Regarding the regeneration of such organs as kidney and liver following damage or partial ablation, another long-lived theory, for which there also was no firm evidence, was that regrowth to normal mass is a response to the extra metabolic effort demanded of the organ remnant. This theory may have originated by false analogy with the well-known reaction of muscle mass to extra usage. The literature on kidney and liver regeneration was massive and today it is still growing. The earlier reviews by Weinbren (*10*), Bucher (*11*), and Bullough (*7*) all showed that contradictory results and contradictory interpretations of similar results were a characteristic of the literature. Also characteristic was the common inadequacy of the experimental techniques, which was too often the result of attempts to obtain maximum results from minimum efforts. The general situation was that the stimulus to regeneration was held to be dependent on the

loss of a mitotic inhibitor, or on the secretion of a mitotic stimulant (sometimes called a "necrohormone," which was a variation on the "wound hormone" theme), or on a combination of both.

It was particularly difficult to separate the significant from the insignificant results, but, from the better work, the following important points did emerge.

1. Each tissue is dominated by its own specific control mechanism. An increased mitotic rate is seen only in tissues that are themselves damaged (*8,12*).

2. The mitotic rate is influenced by some humoral agent which passes through the tissues, as when cell division increases on one side of a mouse ear in response to damage on the other side (*8*), and via the blood, as when the regenerative response to partial hepatectomy is seen also in a distant liver autograft (*13*).

3. The mitotic response is quantitatively related to the amount of damage done. Thus, while a small liver wound causes only a local mitotic reaction (as in a skin wound), when about 10% of the liver is destroyed the reaction spreads to the whole organ and from that point onward the degree of the mitotic response is in direct proportion to the amount of liver destroyed (*14*).

4. A tissue-specific mitotic inhibitor is involved in these reactions. Regarding epidermis, this was established by Bullough and Laurence (*15–17*); regarding kidney and liver, it was first clearly shown by Saetren (*12*).

About a decade ago, this problem of mitotic control was also approached from a theoretical angle. Attempts were made to use Occam's razor to slice through the tangle of evidence and of theory by asking What would be the simplest conceivable control mechanism that could meet the observed requirements? Using epidermis as a model, Iversen (*18*) and Mercer (*19*) proposed a system of mitotic control by the simple negative-feedback action of a tissue-specific antimitotic molecule; Iverson and Bjerknes (*20*) then developed this idea with the use of a computer, and they found that it could be applied successfully to problems of hyperplasia.

THE NEGATIVE-FEEDBACK THEORY

This theoretical negative-feedback control system fitted well with the results of the early experiments of Bullough and Laurence (*17,21*) who found that extracts of epidermis can inhibit epidermal mitotic activity in a tissue-specific manner. The epidermis was quickly shown to contain a tissue-specific mitotic inhibitor which was then named "the epidermal chalone" (*16*). It was suggested that the main source of this chalone may be in the distal cell layers; that it diffuses into the basal cell layer to inhibit mitosis; and that, when the distal cells are sufficiently numerous, the production of new basal cells will cease (*21*). In this way the epidermal thickness, or mass, is maintained from day to day and is restored after epidermal damage.

It was also found that the two stress hormones, adrenaline and the glucocorticoid hormone, greatly augment the antimitotic action of the epidermal chalone (*17,22*). This cooperative action, which is still not understood, was later exploited as an invaluable diagnostic test for the presence of the epidermal chalone in tissue extracts.

About the same time, Rytömaa began work on mitotic control in the granulocyte system, and he too quickly demonstrated the existence of a negative-feedback chalone mechanism (*23*). The postmitotic mature granulocytes dispersed around the body secrete a tissue-specific chalone which passes via the blood to the bone marrow to inhibit DNA synthesis in the progranulocytes. After a sudden loss of mature granulocytes, as during a bacterial infection, the resulting fall in the chalone concentration permits a higher mitotic rate in the progranulocytes.

Since these pioneer studies, a considerable number of other chalone systems have been identified: in the skin—sebaceous glands (*24*), eccrine glands (*25*), melanocytes (*26*), and probably also hair follicle and dermis [*see* (*6*)]; in the blood—erythrocyte system (*27*) and lymphocyte system (*28–30*); and elsewhere—kidney (*31*), liver (*12,32,33*), lung (*34*), and uterus (*35*). It now seems probable that chalone systems will be found to exist in all the mitotic tissues of the body.

THE CHALONE THEORY

A chalone has been considered to be an anti-

mitotic (or anti-S phase) substance which is synthesized within the same tissue on which it specifically acts, which is not species-specific, which inhibits rapidly and reversibly those syntheses on which the mitotic activity depends, and which passes throughout the tissue and into the blood. The various chalones may well prove to be chemically related; present evidence suggests that they are polypeptides or small proteins (*30, 33,36–38*).

A chalone is probably constantly produced within the tissue cells and constantly lost from them into the blood. The intracellular concentration is then determined by the rate of synthesis *plus* the rate of uptake from the environment *minus* the rate of loss to the environment. The role of the chalone is to monitor the state of the tissue from moment to moment, and it could be expected that the speed of the tissue's reaction to any sudden change would be faster if the half-life of the chalone molecule were short. There is, however, no indication at the moment of any special chalone-degrading enzyme system. On the contrary, it is known that chalones, still in the active form, can be recovered from urine (*39*). Thus the indication is that a chalone molecule may be relatively stable and, as described below, it appears that the intracellular chalone concentration is primarily determined by the rate of chalone loss across the cell membrane.

A chalone mechanism has at least three parts: the synthesis of the molecule; the manner of its transport from cell to cell within the tissue and to and from the blood; and the mechanism of the cell response. Chalone synthesis is probably maintained at a relatively constant rate. Chalone transport is evidently controlled by a specific mechanism in the cell membrane, which both hinders the loss of endogenous chalone and promotes the uptake of exogenous chalone. The intracellular chalone concentration is much higher than the extracellular concentration. This membrane mechanism is probably also the basis of chalone tissue-specificity: mitosis must be essentially the same process in all cell types and therefore the antimitotic part of every chalone molecule may be identical; it is the remainder of the molecule that may be recognized and accepted by the tissue-specific cell membrane (*3*).

The manner of the cell response to the chalone may be the most complex part of the whole mechanism, and this is discussed below. At the heart of the mechanism lies the problem of choice: each cell emerging from mitosis must choose whether to synthesize the enzymes necessary for yet another mitosis or whether to synthesize the enzymes on which aging (with tissue function) depends. This is probably a decision that is made at the gene level, and it is common for gene activation or inactivation to be achieved in terms of the intracellular concentration of some type of effector molecule which, in this case, may be the chalone (*3*).

THE CONTROL OF TISSUE MASS

A considerable amount of evidence exists to suggest that the mass of a tissue can be controlled solely by means of a negative-feedback mechanism and that such a mechanism actually exists. However, this suggestion ignores the whole question of postmitotic aging (with tissue function) and, when this is considered, it is immediately found that the control mechanism is more complex than was originally supposed.

Recent analyses (*1,2*) of the situation in epidermis have shown that the epidermal chalone inhibits not only the mitotic rate but also the rate of postmitotic cell aging. Indeed, over the widest range of mitotic activity, these two inhibitions are so precisely matched that the ratio, rate (mitosis):rate (postmitotic aging), = K, a constant. This conclusion also applies to sebaceous glands and to liver, and it is probably universal in mitotic tissues, although clearly the value of K is not always the same. It follows from this that a changed mitotic rate should have no effect on tissue mass, and therefore that the control system is not a simple negative-feedback mechanism.

However, it is also clear that, when the epidermal mitotic rate rises above a certain critical point, the thickness (mass) of the epidermis does begin to increase; a full analysis of this response is given elsewhere (*1,2*). In brief, an increasing mitotic rate depends not only on a shorter mi-

totic cycle but also on an increasing number (or proportion) of the basal epidermal cells becoming involved in mitosis. Epidermal thickness is unchanged up to the point when all the basal cells are mitotic. Beyond this point the increasing number of mitotic cells causes an increasing lateral pressure in the basal layer which then begins to fold or double. This means a greater number of mitotic cells per unit area of skin and, since rate (mitosis):rate (postmitotic aging) = K also implies that number (mitotic cells):number (postmitotic cells) = K′, the number of postmitotic cells per unit area of skin must also increase and the epidermis must thicken.

The same situation is found in sebaceous glands and it is probably universal. However, in these glands, and in other similar tissues, the basal mitotic layer does not fold; it expands like the surface of an inflating balloon. The same sort of response is seen in such target tissues as uterus and vagina after treatment with the appropriate mitogenic hormone.

This control system incorporates two important safety factors: first, however low the mitotic rate may fall (as, for instance, during long-continued stress), no tissue can shrink to less than a certain critical mass; and second, however high the mitotic rate may rise, no tissue can become unduly large because the higher the mitotic rate, the faster the postmitotic cells age and die [*see* (*2*)].

The various body tissues seem to differ in two main particulars—in the inherently different chalone concentrations within their cells, and in the inherently different lengths of their postmitotic aging periods. Regarding the chalone concentration, it appears to be naturally high in such organs as liver and kidney in which mitosis is rarely seen, the rate of postmitotic cell aging is very slow (*40*), and the cell population is functional and almost static in the A_1 phase. The chalone concentration is evidently lower in such tissues as the granulocytic and erythrocytic systems, in which the cells are functional in the dying A_2 phase, and in such external tissues as epidermis, in which tissue function occurs only after cell death. All these tissues depend on a constant supply of dying or dead cells, their mitotic activity is relatively high, and their postmitotic cell life is relatively short.

Regarding inherent differences in the length of postmitotic cell aging, Bullough (*1,2*) has analyzed data showing that the naturally thicker epidermis of the mouse ear is dependent on an inherently longer postmitotic aging process. It is therefore logical to believe that the larger body organs, such as liver, consist of cells with a particularly long postmitotic life expectancy [*see* (*40*)] so that, when mitosis almost ceases in the adult animal, there is in existence a large postmitotic cell mass; a smaller organ, such as a lachrimal gland, could be created by cells with a shorter postmitotic life-span so that, when mitosis almost ceases in the adult animal, there is in existence a much smaller postmitotic cell mass.

On the basis of the two variables, chalone concentration and postmitotic cell life-span, and also such possible complexities as stem cell compartments and hormone-dependent target tissues, all the various mitotic activities and masses of the body tissues and organs can be accounted for.

TISSUE REPAIR AND REGENERATION

One of the most important aids to survival in any long-lived animal is the ability to replace lost cells and so to repair damage. The tissue response to abnormal cell loss may be strictly local (as in wound healing) or it may spread throughout the whole tissue (as in kidney or liver regeneration). All the tissues of the various body and duct surfaces are liable to damage by physical or chemical or biological agents, while internal organs such as liver and kidney are liable to damage by infection or by toxic substances which are either ingested or produced within the body.

The mitotic reaction to local damage involves only the immediately adjacent cells (*8*). In epidermis the cause is twofold: the local cells suffer membrane damage, which results in an excessive loss of chalone (*41*), and the local cell population is decreased, which results in a decrease in the average chalone concentration in that area. The membrane damage must be gentle; if damage is so great that it also involves the cytoplasm and nucleus, a mitotic response may become metabolically impossible.

If the damage is more than local, there comes a point when the mitotic response begins to ex-

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tend throughout the whole surviving cell mass. This type of reaction cannot be studied in epidermis, since the degree of injury needed would be lethal, but it is seen, for instance, in the kidney or liver after extensive cell loss (*14*), in the granulocytic system during bacterial infection when large numbers of mature granulocytes die, and in the erythrocytic system after hemorrhage. Large-scale cell loss evidently leads to a decrease in the blood chalone content, which increases the steepness of the chalone concentration gradient from the remaining normal tissue cells and so leads to an excessive rate of chalone loss. The degree of mitotic response is then in direct proportion to the amount of tissue that has been lost (*14*).

Thus, in all cases the increased mitotic rate following any form of tissue damage may be primarily a function of the increased rate of chalone loss across the cell membranes of the surviving cells.

From what has been said before it is also evident that an increased mitotic rate is always accompanied by an increased rate of postmitotic cell aging, although in some tissues, such as the granulocyte and erythrocyte systems, there are complexities of organization that make this difficult to demonstrate. However, in the case of a single episode of damage, full tissue regeneration is normally achieved before the newly produced cells are due to pass to their earlier deaths. The tissue then recovers its normal chalone concentration and reverts to its normal rate of mitosis and of postmitotic cell aging.

It is only in cases of chronic damage that the role of faster postmitotic cell aging can be appreciated. The chronically increased mitotic rate may lead to hyperplasia, but the increase in mass is strictly limited by the chronically increased rate of postmitotic cell aging. Thus, the situation remains in control with the rate of cell production exactly matched by the rate of cell loss. No growing tumor can result.

THE ACTIONS OF CHALONES

From the above evidence, and from that given by Bullough (*2*) for the epidermis, it is now possible to summarize the ways in which chalones appear to act. Admittedly, the evidence presently available is derived only from a few tissues, but the signs are that the conclusions reached will prove to be generally applicable.

In the first place, it is clear that a chalone inhibits some or all of those syntheses on which the mitotic cycle depends, with the result that this cycle proceeds more slowly (cells may even enter G_0, in which the preparations for mitosis become too slow to be measured) and that, consequently, fewer cells per unit time are able to enter the S phase or mitosis (the inhibition is also felt in G_2). Since both the S phase and mitosis are all-or-none reactions, the necessary syntheses being completed in advance, they are not themselves inhibited by the chalone.

In the second place, a chalone inhibits the passage of a postmitotic cell through the A_1 phase of aging [*see* (*2*)]; with high chalone inhibition, as in the liver, the tissue cells remain almost permanently in A_1. The later A_2 phase of aging is evidently typified by a nonfunctional nucleus (cell survival depending on preformed mRNA); no data exist but it seems possible that this phase may not be affected by the chalone.

The third action of a chalone relates to the choice taken by the dichophase cell—either to enter the mitotic cycle once more or to enter the aging pathway. The evidence is that the decision depends on the chalone concentration. There are two important points of evidence: first, it is known that a cell in A_1 (as in the stratum spinosum of wounded epidermis or in the cells in the liver remnant after partial hepatectomy) reverts to mitosis if the intracellular chalone concentration falls to a low enough level (*41*); and second, it is known [*see* (*2*)] that when (as in wounded epidermis) the chalone concentration is suddenly decreased a greater than normal proportion of the dichophase cells reenter the mitotic cycle. The problem posed by this second response is considered elsewhere (*2*) but it seems safe to conclude that the chalone concentration within the dichophase cell is the deciding factor determining the fate of that cell. If the concentration is below a certain critical level, the cell prepares for mitosis; if it is above that level, the cell enters the aging pathway.

It is important to emphasize that these chalone

actions do not depend on any general metabolic inhibition; they relate only to specific aspects of the cellular activities.

CHALONES AND CANCER

It has always been obvious not only that the mitotic activity of any tissue is closely regulated but that, in Iversen's (*42*) words, "in malignancy something is wrong with this regulation." It has also always been obvious that the nature of cancer is most unlikely to be understood until the mechanism controlling normal tissue mass has itself been elucidated.

Obviously, much further work remains to be done but already a number of important conclusions are emerging [*see especially* (*1,2,43–49*)]. The main points may be summarized as follows: in a wide range of experimental tumors, the cells continue to produce considerable quantities (perhaps even normal quantities) of the chalone of their tissue of origin, and they also continue to respond by mitotic inhibition to this chalone; in all tumors so far studied, the postmitotic cells continue to die in the normal manner via the aging pathway (with or without normal tissue function) so long as the blood supply remains adequate; the evidence now available suggests that tumor cells are characterized by an abnormally low chalone content, which may be due to a too-rapid chalone loss across an abnormal cell membrane; tumor growth therefore results in an increasing chalone concentration in the body so that first the mitotic activity of the tissue of origin is inhibited and later the mitotic activity of the tumor itself is inhibited; consequently, tumor growth commonly follows a sigmoid curve which may either plateau before death of the host, so that the tumor becomes chronic, or may fail to reach its plateau before death, so that it is lethal (or having first become chronic it may later progress to a more active form and so become lethal).

All growing tumors are in a state similar to that of a normal tissue that is *in the* process of becoming hyperplastic. Bullough (*2*) has described how at this time the rate of production of mitotic cells exceeds the rate of production of postmitotic cells. In an ordinary tissue these two rates come back into balance as soon as a new steady state (normal or hyperplastic) is reached. In a tumor this does not happen unless, as in a chronic tumor, the chalone concentration rises to a high enough level.

In the last few years the question has repeatedly arisen as to whether the chalones could have any therapeutic value in the treatment of cancer. Extensive studies have shown, both in vivo and in vitro, how the appropriate chalone can inhibit mitosis in a number of epidermal carcinomas (*50–53*) and melanomas (*54,55*) (figs. 1–4, *see* end of paper), in granulocytic leukemia (including human spontaneous granulocytic leukemia: *56–58*), in lymphocytic leukemia (*28,30,59*), and in other tumor types [*see* (*35,46–48*)]. One great advantage of tumor treatment by chalones is the strict tissue and tumor specificity of the antimitotic action. However, a pessimistic view of the prospects has been expressed by Iversen (*60*) who believes that a chalone, acting in the way it is known to do, cannot be expected to destroy a tumor. In this, Iversen is certainly theoretically correct; the best that could reasonably be expected would be that a chalone might stop further tumor growth and cause regression.

The actual evidence, however, leads to a more optimistic view, and it is already clear, at least in some cases, that chalone treatment can lead to total tumor destruction. The most dramatic experiments so far are those of Rytömaa and Kiviniemi (*57,58*) who obtained permanent cures in cases of rat granulocytic leukemia (text-figs. 1,2). The probable explanation has been given by Rytömaa (*61*) who has emphasized that tumors, unlike normal tissues, do not live and grow in ideal conditions. They must always fight against adverse circumstances of which perhaps the best known are the common inadequacy of the blood supply [*see* (*49*)] and the common induction of an immune reaction [*see* (*62*)]. The rate of growth of a tumor, which is often remarkably slow, is therefore a function of the increased mitotic potential minus the decreased rate of mitosis and the increased rate of cell death that result from the adverse circumstances. If the mitotic potential is decreased by chalone treatment to the point at which the adverse circumstances predominate the tumor may indeed disappear.

TEXT-FIGURE 1.—Effect of repe
cytic chalone on rat chl
Harding-Passey melanoma (
accompanied by ulceration
the wound.

This is the only obvio
one could be expected to destroy a tumor, and interestingly it is the precise way in which such an antibiotic as penicillin destroys an infection. Penicillin merely inhibits bacterial multiplication, and the body's own defense mechanisms are then able to predominate.

CHALONES AS CHEMICAL MESSENGERS

The first "chemical messenger" was extracted in 1902 by Bayliss and Starling (*63*). Four years later, at a meeting of the Gesellschaft Deutscher Naturforscher und Ärzte held in Stuttgart, Starling (*64*) proposed that such a substance should be called a hormone: "ich schlage vor, diesen Substanzen einen eigenen Namen zu geben, und ich werde sie deshalb fernerhin in diesem Vortrage als Hormone (von ὁρμάω = ich reize oder rege an) bezeichnen" (text-fig. 3). Later in the same speech he referred also to "hemmende Hor-

TEXT-FIGU
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TEXT-FIGURE 3.—Professor E. H. Starling who, in 1902 with Dr. W. M. Bayliss (*63*), extracted the first "chemical messenger," which he later (*64*) called a "hormone." (From Starling's Principles of Human Physiology, edited by C. Lovatt Evans. London, Churchill, 1941. Courtesy of Churchill Ltd., London.)

TEXT-FIGURE 4.—Professor Sir Edward A. Schäfer who, in 1916 (*65*), proposed that the word "chalone" should be used to describe an inhibitory "chemical messenger." (Courtesy of The Royal Society, London.)

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mone, which excites to increased activity."

This proposal was never accepted and, today, a hormone may be an inhibitor or a stimulant. Thus, in 1962 the word "chalone" was free to be adopted by Bullough (*16*) for the type of tissue-specific antimitotic substance which was at that time being extracted from the epidermis. It is a curious coincidence that the first chalone preparation was made in London (*15–17*) in a laboratory neighboring that in which, exactly 60 years earlier, the first hormone preparation had been made (*63*).

The chalones are obviously internal secretions with a regulatory function, and there can be no question that they form part of the science of endocrinology. In this science there are now three main subdivisions: the chalones which are produced within each tissue to maintain the mass and therefore the functional ability of that same tissue; the hormones which are produced within one tissue to determine the functional ability, and commonly also the mass, of some other tissue; and the pheromones which are produced within one individual to control the functional ability, and commonly also the mass, of a tissue in another individual.

SUMMARY

In the past the regulation and regeneration of tissue mass have commonly been considered in terms of mitotic control, and this has led to the theory of the negative-feedback chalone control mechanism. Tissue-specific antimitotic chalones have been found in all tissues in which they have been sought.

However, a chalone inhibits cell gain and cell loss in equal degree and therefore the mechanism is not a simple negative feedback. The manner in which epidermal mass is determined by the epidermal chalone mechanism is described; it is probable that the conclusions reached apply equally to other tissues.

The decrease in the chalone concentration which leads to a raised mitotic rate after tissue damage seems primarily due to an increased rate of chalone loss across the cell membranes.

Some tissue control systems show variations on the common theme: in hair roots the chalone mechanism oscillates to produce the hair growth cycle; in some tissues the chalone control is modified by hormone action; while in other tissues new cells are also recruited from relatively undifferentiated stem cell populations.

All tumors so far studied show apparently normal patterns of chalone production and response. Their cells may have a low chalone content due to a high chalone loss, similar to that which occurs in damaged cells. Commonly, the tumor growth rate is low, since the mitotic advantage is offset by adverse conditions. At least in some cases, an increasing chalone concentration, whether occurring naturally or artificially, can so decrease the mitotic rate that the adverse conditions can inhibit or destroy the tumor.

The endocrine system comprises: the chalones, each of which regulates the tissue within which it is produced; the hormones, each of which regulates a different tissue from that in which it is produced; and the pheromones, each of which is produced in one individual to regulate a tissue in another individual.

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Figure 1.—Harding-Passey melanoma. Section of whole tumor, showing peripheral growing region and central mass of dead cells (*49*). Figure 2.—Mitoses in outer growth region, postmitotic cells synthesizing melanin in medial region, and decomposed dead cells in central region. Figure 3.—First phase of chalone-induced melanoma regression, showing inhibition of mitosis in outer cells which then turn to melanin synthesis and death. Figure 4.—Later phase of regression, showing melanin synthesis in outermost cells preparatory to the death of the whole melanoma.

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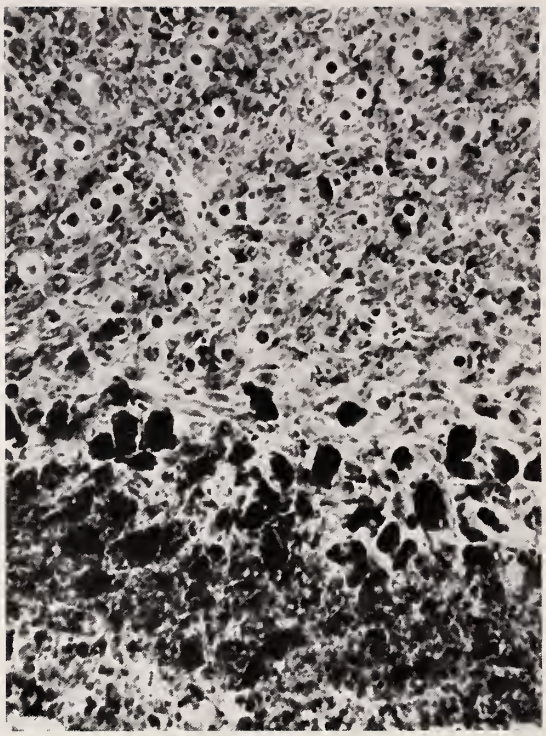

THE EPIDERMAL CHALONE

An Assay System for Humoral Growth Factors [1, 2]

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SUMMARY—Explants of organs from newborn and adult rats have been successfully maintained in glass bottles kept under continuous agitation on an inclined turntable rotating at 25 rpm. In cultures of newborn organs the rate of tritiated thymidine incorporation appears to bear no relationship to the rate of cell proliferation and may be influenced more by the intracellular thymidine pool concentration. In mature lung, and possibly other organs, thymidine incorporation correlates well with the rate of entry into DNA synthesis and mitosis. A combination of this culture technique and the isotope method provides a straightforward procedure for analyzing the action of humoral factors which control cell division.—Natl Cancer Inst Monogr 38: 19–28, 1973.

THERE IS evidence that the growth of many organs is controlled by chemical factors which regulate the rate of cell division and which are capable of diffusion via the extracellular milieu or of circulating in the blood (*1*). Such humoral growth regulatory factors, established or postulated, include hormones (*2*), chalones (*3*), products of tissue function (*4*), tissue breakdown products or "wound hormones" (*5*), and immunological factors (*6*). In view of the widespread occurrence of such substances it is important to describe biological systems in which their action can be studied in a detailed and reproducible manner. There is a limit to which in vivo systems can be used for this purpose since, apart from practical considerations of the large number of individual animals required for statistical purposes, there is the more fundamental disadvantage of having to decide whether the effects obtained are due to the direct action of test substances upon the tissue in question or to intermediate mechanisms. There is obviously a strong argument for using in vitro culture systems in this situation.

The second practical consideration is the choice of criteria for measuring growth. Particularly in such phenomena as repair and regeneration, the tissue responds very rapidly to changed circumstances, and cybernetic principles indicate that to achieve such fine control the messenger substances must have a rapid rate of turnover. It is unlikely, therefore, that test substances added to the culture system would be active sufficiently long enough to produce any significant alteration in the net mass of tissue present. Since cell division is of major importance in most growth phenomena it is advisable to use cell proliferation parameters in which relatively short-lived changes can be readily detected.

Having decided in principle that the analysis of cell proliferation in vitro provides a useful assay system, the detailed choice of techniques must now be considered. In cultures of isolated cell types, many properties of the differentiated cell may be rapidly lost (*7*)—e.g., mouse prostate in organotypic culture responds to testosterone, which is a growth-control factor for this organ,

[1] Presented at the First Symposium of the International Chalone Conference, Brook Lodge, Augusta, Mich., June 5–7, 1972.

[2] Supported by grant G–970–408B from the Medical Research Council of Great Britain.

[3] With the technical assistance of John Walton whom we thank for his help in developing the techniques here described.

while outgrowths of prostatic epithelium do not (*8*). Organ culture therefore seems more suited to the type of experiment under consideration and has been used extensively in our laboratory for studies on growth control (*9–11*). The disadvantage of this method is that the classic techniques require elaborate apparatus (*12*), which imposes practical limitations on the size of the experiment.

In this paper we describe a much simplified technique which permits considerable improvement in experimental design. Measurement of cell proliferation rates in our previous studies has mainly depended on counting mitotic and labeled cells in histological and radioautographic preparations (*13*), which is very time-consuming. The incorporation of labeled thymidine into cells, which may be used as an index of DNA synthesis, is a parameter which can be measured with automatic counting apparatus. A second feature of the present paper is to examine the correlation between thymidine incorporation and the rate of DNA synthesis calculated from radioautographic preparations.

MATERIALS AND METHODS

Source of tissue and culture technique.—In all experiments we used organs from rats, chiefly of two ages: 3 days and 6–9 weeks. In the case of 3-day-old material we selected only healthily growing animals (weight, approximately 9 to 10 g) and found this to be of great importance in obtaining standard results. Organs dissected from the animal were cut into explants of approximately 1.5 mm^3 and stored temporarily in a pool of medium; improvements in the cutting technique will be described below. As a basis for comparison we used Trowell's organ culture technique in which explants, usually in groups of 10–15, are placed on a strip of lens paper supported by a grid of titanium mesh in a glass silica dish containing 5 ml of medium [Trowell's T8 (*12*) with 20% fetal calf serum]. The culture dish is placed inside the standard aluminum organ culture chamber which is gassed with 95% O_2 and 5% CO_2 before incubation at 36 to 37° C.

Histology and radioautography.—To arrest mitosis at metaphase, Colcemid was added to the medium, at a final concentration of 0.4 μg/ml, 2 hours before the end of the experiment. For radioautography, tritiated thymidine (^{3}H-TDR) was added to the culture medium at a final concentration of 1 μCi/ml, usually 1 hour before the end of the experiment. Explants were fixed in Carnoy's fluid, sectioned at 6μ, and stained in hematoxylin and eosin for mitotic preparations or coated with Kodak AR-10 stripping film, exposed for 1 week, and stained through the emulsion with hematoxylin for radioautography. Mitotic or labeled cells were counted at a magnification of 1000 using sampling procedures described elsewhere (*13*), and the mitotic incidence (MI) or labeling index (LI) was expressed as the proportion of counts per 10^5 cells.

Isotope counting methods.—DNA was extracted from explants by a modified Schmidt-Thannhauser technique (*14*). Tissues were incubated overnight at 37° C in KOH, cooled, and mixed with cold perchloric acid (PCA), after which the precipitate was incubated with PCA at 65° C. The total DNA content of the resulting supernatant was estimated by using Burton's diphenylamine reaction (*15*), while part of the DNA sample was mixed with a toluene-based scintillation fluid and the rate of emission of β-particles measured in a Beckmann LS-100 liquid scintillation counter. The rate of incorporation of thymidine (RIT) is given by the specific activity of labeled DNA, which was expressed as counts per minute/μg of DNA.

Measurement of metabolic rate.—Utilization of glucose by the cultures was measured by taking 0.2-ml samples of the culture medium and assaying them enzymatically with peroxidase and glucose oxidase (*16*). Production of lactate was assayed by using lactate dehydrogenase (*17*) in the form of a test kit (Boehringer Corp.). Results of both of these tests were standardized by using a method for determining the total organic content of the explants (*18*).

DEVELOPMENT OF TECHNIQUES

Culture Method

Evidence from a large number of experiments showed that normal-sized explants from a va-

riety of newborn and mature rat organs could be cultured successfully submerged in the medium in 50-ml medicine bottles, provided that the bottles were gassed with O_2 (containing 5% CO_2) and mechanically agitated. We have designed a machine (fig. 1) which consists of an inclined variable-speed turntable containing slots for the culture bottles. In practice, a speed of 25 rpm appears to be optimal because at less than this the explants stick to the glass or clump together. The only organ tested so far which is not amenable to this technique is the prostate, explants of which stick together due to mucous secretion.

An experiment was performed as a critical comparison of this method with the Trowell (*12*) culture technique. Lung explants from 3-day-old and 6-week-old rats were maintained in bottles and in Trowell chambers; samples of medium were withdrawn for metabolic tests after 12, 24, 48, and 72 hours. At the end of the experiment, histological preparations were made, from which mitotic counts were estimated. The data are summarized in table 1. For both newborn ($P<0.01$) and for adult material ($P<0.05$), the bottle technique gave a significantly higher mitotic index than did Trowell's technique. For both newborn and adult material, glucose consumption was higher and lactate production was lower in bottles than in Trowell cultures, although the differences were small. The experiment showed that the bottle method was at least as good as the Trowell technique in respect of rates of cell division and metabolism. Maintaining the explants submerged in the medium evidently does not encourage an increased rate of anaerobic respiration.

TABLE 1.—*Comparison of rotated bottle and Trowell chamber culture methods**

Organ	Method	Mitotic index (per 10^5 nuclei)	Total organic matter (TOM) (mg)	Glucose utilization (mg/mg TOM)	Lactate production (mg/mg TOM)
Newborn rat lung	Bottle	414†	3.50	2.75	0.34
	Chamber	256†	3.00	3.33	0.36
6-week-old rat lung	Bottle	188‡	5.05	3.60	0.28
	Chamber	110‡	4.05	3.85	0.30

*Material cultured for 3 days.
†For difference between methods, $P<0.01$.
‡For difference between methods, $P<0.05$.

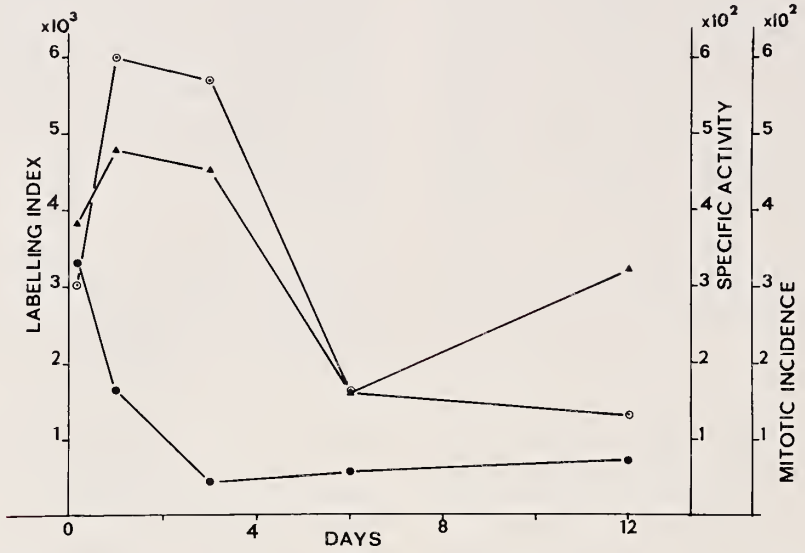
Explants of rat lung have been successfully maintained in bottle cultures for up to 12 days, the longest period so far tested. Adhesion of explants to the glass or to each other frequently occurs during prolonged culture and has a deleterious consequence in that necrosis occurs in the center of adhering explants, probably due to anoxia. Treatment of the bottles with silicone prevents explants sticking to the glass, and we are currently testing the effect of adding methylcellulose to the medium, since this is used in suspension cultures to prevent adhesion of cells (*19*).

Two mature mammalian organs reported as being difficult to maintain in organ culture are liver and kidney. Early in our work we found that the histological preservation in explants of these organs was very variable: Tissue near the cut edges of the explant was often completely necrotic while in untraumatized regions the cells appeared to be healthy (fig. 2). Therefore, our explant cutting technique was modified. We now hone our cataract knives to razor sharpness before each experiment and, instead of cutting up the tissue directly on a glass plate, we now place it on a strip of sterilizable adhesive tape covering the glass surface; this has the twofold advantage of conserving the knife edge and of permitting a clean cut without tearing or shredding the tissue. By attention to such small details we now obtain very good results with explants of mature rat kidney and liver (fig. 3). There is a vast literature dealing with the phenomenon of compensatory

growth in these two organs, and we believe that a satisfactory organ culture system will prove useful in the further analysis of the regulatory mechanisms involved.

Incorporation of Thymidine and Rate of Cell Proliferation

In explants of 3-day-old rat lung, RIT decreases between 4 and 24 hours (text-fig. 1), and this phenomenon also occurs in newborn rat kidney. Corresponding values for MI and LI do not show a similar decrease over the same period, and it is evident that RIT, in this material, does not provide a reliable index of cell division rate. From what has been said in the previous section concerning the metabolic rate and long-term survival of newborn lung explants, it is obvious that the decrease in RIT cannot be explained on the basis of decreased cell viability.



TEXT-FIGURE 1.—Comparison of MI, LI, and RIT in cultures of newborn rat lung. —●— = RIT, measured by specific activity of DNA (counts/min/μg DNA) after incubation with ^{3}H-TDR; —⊙— = MI, number of arrested metaphases per 10^5 nuclei after 2 hours of Colcemid treatment; —▲— = LI, number of labeled nuclei per 10^5 nuclei after 2 hours of ^{3}H-TDR treatment. *Note* the correlation between MI and LI and the lack of correlation between LI and RIT.

By contrast, in mature rat lung there is a much closer correlation among RIT, MI, and LI. Over the first few hours after explantation, RIT, MI, and LI decrease slightly but after this they recover, reaching a maximum at 48 hours (text-fig. 2). In this respect they resemble the behavior of MI and LI as described in other organs following explanation (*10,20*). It therefore appears that the usefulness of RIT as a method of measuring cell proliferation rate may vary according to the age and possibly the type of the organ.

While the object of this paper is to describe successful methods rather than to analyze failures, it is appropriate to give some brief explanation for the decrease in RIT observed in newborn explants. If the explants are transferred to fresh medium after 24 hours, there is an increase in RIT, although this does not attain the high values observed at 4 hours after explantation. The decrease at 24 hours therefore appears to be due to both changes in the medium and changes in the tissue. Further analysis of the nature of the change in the medium shows that the decrease in RIT is not due to pH changes, depletion of essential components of the medium, or accumulation of enzymes which break down ^{3}H-TDR. Addition of unlabeled TDR to the medium, at appropriate concentrations, decreases RIT due to a competition effect, and the most likely hypothesis to explain the low value for RIT ob-

TEXT-FIGURE 2.—Comparison of LI, MI, and RIT in cultures of adult rat lung. —●— = LI; —▲— = MI; —⊙— = RIT. For definition of parameters, *see* text-figure 1.

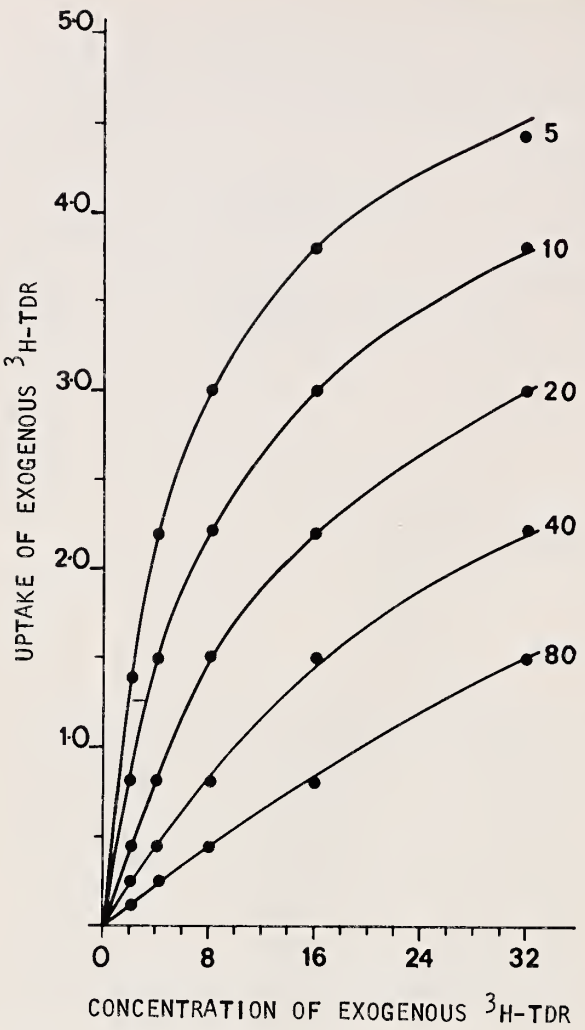
served at 24 hours is that the explants liberate TDR into the medium and this competes with the label.

Concerning the nature of the changes intrinsic to the tissue, we examined the possibility that after explantation the tissue might show an increasing preference for the normal biosynthetic pathway for DNA replication at the expense of the salvage pathway through which TDR is incorporated (*21*). Blockage of the normal pathway by amethopterin (*22*) did not lead to an increase in ^{3}H-TDR incorporation, and this hypothesis appears to be incorrect.

A second possibility tested was that the intracellular pool of TDR might be depleted immediately after explantation and that, as a consequence, there would be little competition with exogenous ^{3}H-TDR which would be readily incorporated. The direct assay of TDR presents a number of difficulties and we therefore used a method for the indirect estimation of relative pool size based on the theoretical premise that the correlation between RIT and ^{3}H-TDR concentration tends to become more linear as the degree of competition increases (text-fig. 3). When explants of newborn lung were cultured in a graded series of ^{3}H-TDR concentrations, the graph of values for RIT at 4 hours was distinctly curved whereas at 24 hours it was more rectilinear (text-fig. 4); in this respect it resembled the theoretical curves in text-figure 3. The difference between the two curves was also obtained when fresh medium was used for the 24-hour series and therefore is not dependent on changes in the medium between 4 and 24 hours. These experiments support the view that the lack of correlation between LI and RIT in newborn material is due to changes in the intracellular thymidine pool.

DISCUSSION

It has generally been found that central necrosis in organ culture can be avoided by restricting the size of the explants to 1 or 2 mm^3 and by maintaining them supported at the surface of the medium in an atmosphere of high oxygen concentration. In view of the complicated procedures used for supporting the explants, which include plasma clots, floating rafts, and metal grids, surprisingly little work has been done on alternative methods. The simple method used by Parker (*23*) for culturing adult rabbit spleen explants submerged in shallow medium is suited to other organs (*24*) but has not been widely used. It has been stated (*25*) that, if explants are mechanically



TEXT-FIGURE 3.—Theoretical curves for RIT (uptake of exogenous ^{3}H-TDR) at different concentrations of labeled thymidine added to the medium (concentration of exogenous ^{3}H-TDR). Separate *curves* are for different concentrations of endogenous TDR (intracellular thymidine pool). Assuming a total utilization of 5 units (all units arbitrary) under all conditions the graphs show: 1) a competition effect—the higher the intracellular pool concentration, the lower the incorporation of exogenous label; and 2) variation in rectilinearity—the correlation between uptake of exogenous ^{3}H-TDR and concentration in the medium becomes more rectilinear as the intracellular pool size increases.

agitated, they rapidly disintegrate, and a number of ingenious appliances have been devised to maintain a slow circulation of medium around cultured explants. The idea that movement may cause disintegration appears to be quite unfounded in the light of the work described above and of other studies in our laboratory using a wide variety of organs including thymus, spleen, gut, adrenal, and salivary gland.

The importance of delicate handling of the tissues, mentioned by other authors (*12, 19*), is emphasized in the present study, particularly in the case of the liver which may be rendered completely necrotic if unduly traumatized. Mechanical tissue slicers such as are used for preparing large numbers of tissue samples for biochemical work are not recommended as a means of preparing organ culture explants.

While the rate of DNA synthesis can be analyzed very rapidly by using liquid scintillation counting, it cannot be assumed that the values obtained necessarily bear any relationship to the rate of cell replication. Such lack of correlation was apparent in newborn lung and kidney, although, by contrast, thymidine uptake in adult lung cultures corresponded well with the rate of cell proliferation. Also, recovery in the rate of thymidine uptake by 24 hours after explantation has been observed in liver explants from rats 2–3 weeks old. It therefore appears that isotope methods can provide a very rapid means of analyzing the cell proliferation rate, provided that one chooses an organ suited to this method.

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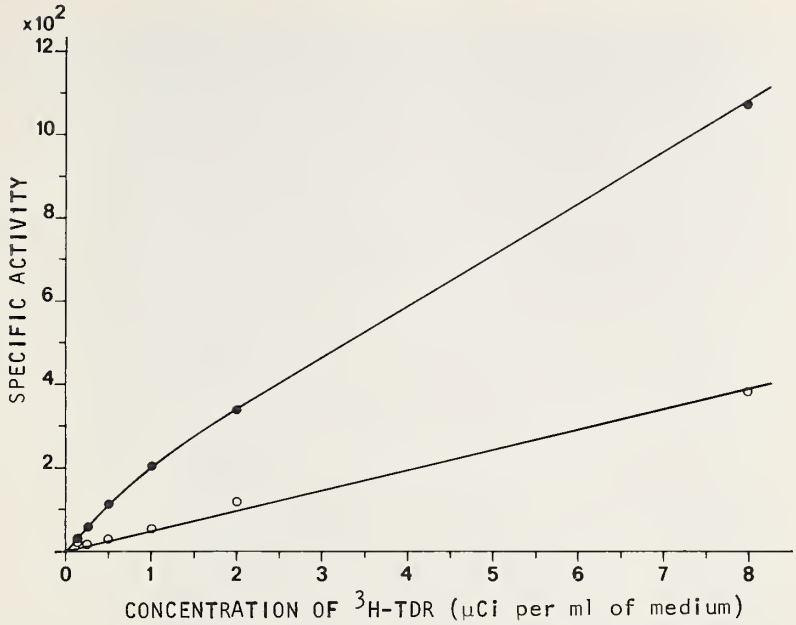

TEXT-FIGURE 4.—Experimental curves for uptake of exogenous ^{3}H-TDR (specific activity of DNA or RIT) at different concentrations of labeled thymidine added to the medium. —●— = 4-hour culture; —○— = 24-hour culture. Comparison with text-figure 3 suggests that the intracellular pool is higher at 24 than at 4 hours, leading to lower values for RIT (competition effect) and a greater rectilinear correlation between RIT and exogenous label concentration.

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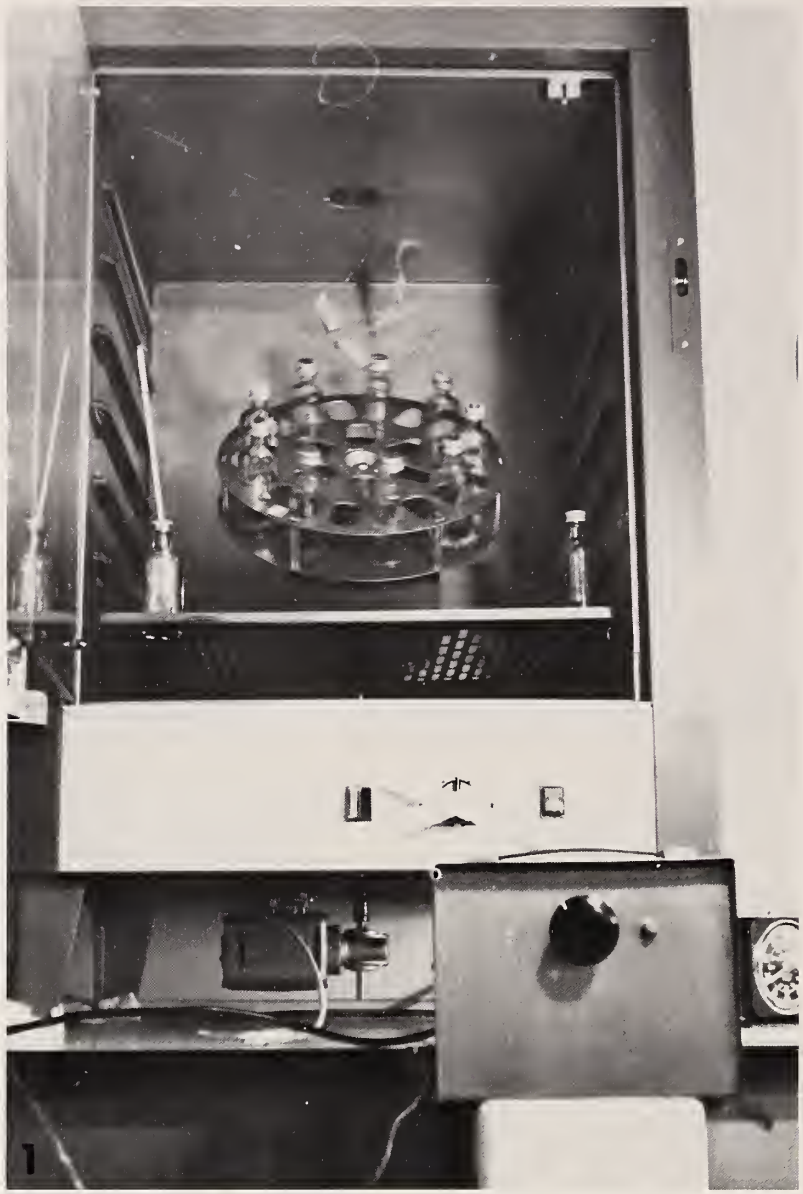

FIGURE 1.—Inclined rotary turntable for organ culture. The turntable with culture bottles in place is situated inside the incubator, and the motor can be seen mounted on the underside of the incubator. Also shown is the separate electronic control unit.

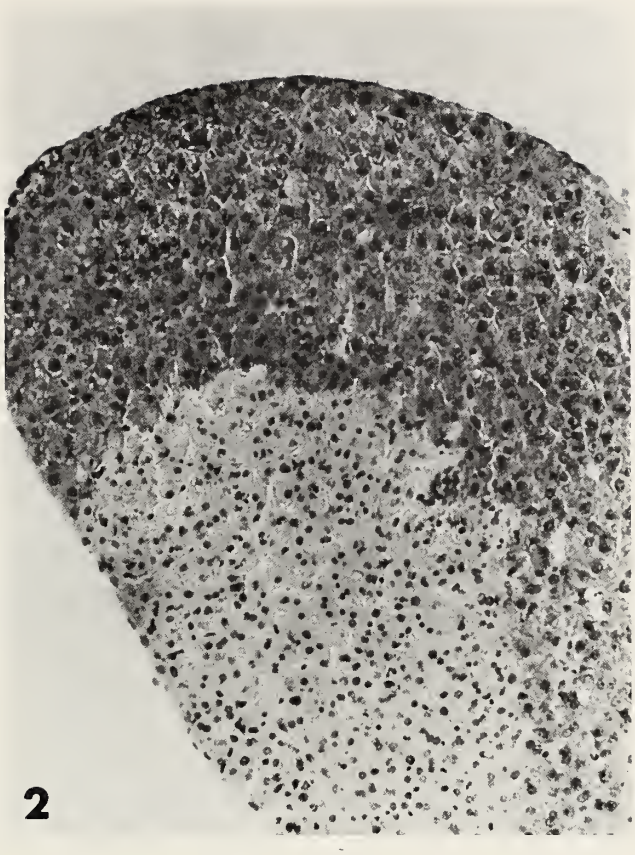

FIGURE 2.—Newborn rat liver cultured for 12 hours. Traumatized region is surrounded by area of necrotic tissue. Hematoxylin and eosin. × 250

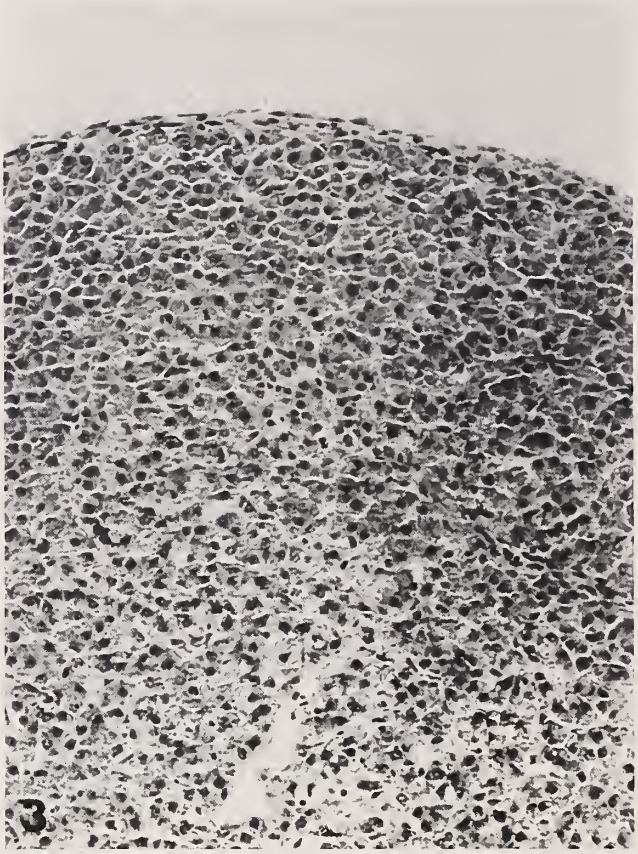

FIGURE 3.—Newborn rat liver cultured for 48 hours. Explants were cut so as to minimize mechanical trauma, and there are no areas of necrotic tissue. Hematoxylin and eosin. × 250

Description of Growth Phenomena and the Formulation of Growth Control Models [1, 2]

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SUMMARY—Developmental and compensatory growth of animal organs is too complex to be explained solely in terms of the homeostatic regulation of cell division by chalones. Examples of phenomena which suggest that existing concepts of growth control should be extended are as follows. Compensatory growth of some organs involves not only the proliferation of functional cells to produce more of the same type, a process which could be controlled by chalones, but also the replenishment of proliferative populations by differentiation from other cell types. The lung is an example of the latter process which may depend on morphogenetic interaction between tissues. Compensatory growth is commonly assumed to depend on systemically circulating tissue-specific control factors. However, marked increases in the rate of blood flow may occur in some organs after damage or extirpation, and it is suggested that the stimulus to growth may depend not on alterations in the concentration of a systemic factor but on an increase in the rate of clearance of a locally diffusible factor. Moreover, since the changes in vascular flow are highly specific, there is no need for the control factors to be tissue specific in their chemical structure. The effects of many chalones have been described in terms of only one stage in the cell cycle, but the idea that the rate of cell proliferation may be determined by regulation at one critical point in the cycle is untenable. It follows either that chalones must act at more than one stage in the cell cycle or that subsidiary coordinating mechanisms must exist. Evidence for these alternatives is lacking.—Natl Cancer Inst Monogr 38: 29–36, 1973.

ONE WELL-ACCEPTED principle of scientific investigation is to start by testing the least complex hypothesis, complexity being generally defined in terms of the number of interacting factors involved in the phenomenon being studied. However, through the evolutionary process, living organisms deliberately chose complex systems of interacting factors, since these provide the high degree of flexibility and control required if the behavior of the organism is to be adaptable. In testing the simplest hypothesis first, the biologist should realize that he is doing this because it is methodologically correct and not because the hypothesis itself is the one most likely to be correct.

That the rate of cell division may be controlled by tissue-specific inhibitors or chalones is a simple hypothesis which lends itself to experimental verification, but there are many growth phenomena which cannot be explained solely on the basis of chalone action. In this paper we discuss some of the problems which must be understood if the idea of chalone action is to be incorporated into

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[1] Presented at the First Symposium of the International Chalone Conference, Brook Lodge, Augusta, Mich., June 5–7, 1972.

[2] Supported by grant G–970–408B from the Medical Research Council of Great Britain.

[3] With the technical assistance of Mr. John Walton.

a more complex overall description of growth regulation.

RELATIONSHIP BETWEEN GROWTH AND MORPHOGENESIS

During development, an organism increases in both complexity and size. Despite this obvious fact, until recently embryologists have been preoccupied with the study of cell differentiation and morphogenesis, and the ordered growth of the embryo has been regarded as little more than an inevitable consequence of programmed cell division. At present the biologist who studies growth control in mature tissues is frequently in the converse position of paying too much attention to cell proliferation and disregarding the importance of cell differentiation. The phenomenon of compensatory growth, in which new functional tissue is produced to make good the loss sustained through accidental damage, has become a favorite subject for the study of growth control, and the liver has received particular attention.

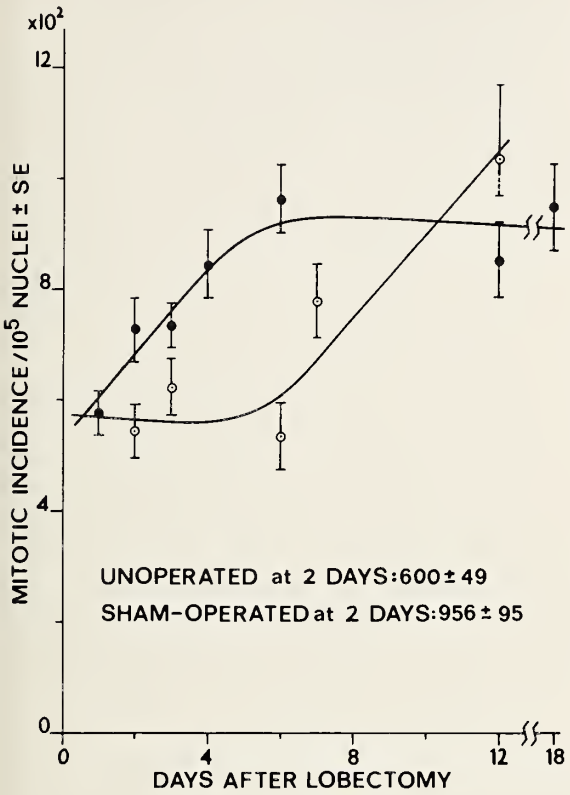
One of the number of reasons for this is that the cell proliferation response in the liver is dramatic and well defined. Approximately 16–18 hours after partial hepatectomy, the rate of DNA synthesis in the liver begins to increase, reaching a maximum after 20–24 hours, while the maximum rate of mitosis itself is observed 6–8 hours after maximum DNA synthesis. Maximum rates of DNA synthesis and mitosis are sustained only for a short period, after which they decrease sharply (*1*). A number of other tissues exhibit this type of response (*2*) which has thus become accepted as a standard pattern for compensatory growth. The analysis of how this response is initiated and then cancelled has tended to divert attention away from other factors of equal importance in growth control, and we therefore will describe some aspects of compensatory growth in the mammalian lung in more detail because, in this organ, cell proliferation and differentiation appear to be inseparable.

In the adult lung there is a rapid rate of turnover of alveolar cells (*3, 4*) although in most species the fully grown animal cannot regenerate new functional alveolar tissue (*5*). This is an interesting problem in itself, since we might expect that a tissue with the capacity for renewal might also be able to produce excess cells for the purpose of regeneration. In immature animals (*5*), however, and in animals such as the rat and Amphibia which continue to grow even in adult life (*6, 7*), a true compensatory response does occur after tissue damage. Romanova and her colleagues have shown that both in Amphibia (*8*) and in the rat (*9, 10*) there is a proliferative response in alveolar cells, with a pattern closely resembling that described in the liver (*1*), and this is eventually followed by a restoration of the mass of the organ (*11*).

Restoration of lung mass following partial extirpation is inhibited by implanting inert substances into the thoracic cavity (*6*) and we have shown that this may be due to interference with the proliferative response of alveolar cells (text-fig. 1) (*12*). This observation, together with the fact that unilateral collapse of lung without loss of tissue causes compensatory proliferation, suggests that the direct stimulus to compensatory growth is not loss of tissue mass per se. The phenomenon which most attracted our attention in these experiments, however, was that other cell types showed a more obvious response to loss of total lung tissue than did alveolar cells. Bronchial epithelium was stimulated to a marked degree (fig. 1), and a high rate of mitosis was observed in interstitial cells adjacent to the pleural surface (fig. 2). Healing of surface wounds in the lungs of cats was followed by differentiation of bronchial tissue into new alveoli which at early stages resembled the alveoli of fetal lungs (*13*).

Our experiments therefore support the view that compensatory growth in this organ may entail the proliferation of existing alveolar cells together with the production of new alveoli from bronchial epithelium and interstitial tissues. The mechanism of differentiation may be similar to that in the embryonic lung where growth is regulated by an inductive interaction between epithelial and mesenchymal cells, each of which produces a diffusible factor which stimulates the growth of the other (*14*). The interstitial cells, proliferation of which has been described above, may have an important function in inducing the differentiation of bronchial into alveolar epithelium, and the interstitial cells themselves are

probably replenished from a differentiating population of cells derived from circulating blood monocytes (*15*).



TEXT-FIGURE 1.—Effect of lobectomy and other experimental treatments on mitotic incidence (MI) in contralateral lung. —●— = MI in right lung after removal of left lung; —⊙— = MI in right lung after removal of left lung and implantation of sponge into left lung cavity. Unoperated animals received no experimental treatment: MI measured in right lung. Sham-operated animals had collapse of left lung without removal or damage. Data show that removal of the left lung stimulates cell division in the undamaged right lung and that this response is delayed by sponge implants. Collapse has the same stimulatory effect as removal.

Inductive interactions are known to play a part in growth homeostasis in other adult tissues. For example, the implantation of plastic films between epidermis and dermis may cause neoplastic proliferation of the epidermis (*16*); the dermis from sites treated with chemical carcinogens may induce proliferation in pieces of untreated epidermis grafted to the same site (*17*). More direct evidence for interaction between dermis and epidermis comes from recent work on chemical growth-control factors in the skin (*18*). Morphogenetic interactions probably occur even in the regenerating liver, since the proliferative response is accompanied by the differentiation of new lobules (*2*).

Most organs comprise a number of cell types and it is difficult to envisage how the exact proportion of such elements could be maintained during compensatory growth solely by each tissue monitoring itself by a negative-feedback system mediated by tissue-specific inhibitors. Morphogenetic interactions between tissues, such as described for the embryonic lung (*14*) and here proposed for the regenerating lung, would provide an extra degree of control. A further possible reason for morphogenetic processes in adult organs concerns the detailed kinetics of the cell renewal process. Many mature mammalian cells survive no longer than 50 divisions in culture and this may be a natural limit imposed by aging (*19*). However, in a large number of tissues there is a rapid rate of turnover (*20*) and if this was sustained simply by the production of like cells from like, the apparent limit would be exceeded in less than the natural lifespan of the whole organism. It can be shown, nevertheless, that, taking the organism as a whole, the total complement of cells produced in a lifetime can be produced by less than 50 cell divisions (*19*). A morphogenetic process could provide a way of replenishing cell populations with a high rate of division through the differentiation of cells from relatively static populations.

COMPENSATORY GROWTH: DOES IT IMPLY TISSUE-SPECIFIC SYSTEMIC FACTORS?

In a number of paired or multiple organs, best typified by the kidney, removal of one organ is followed by compensatory growth in a homologous organ elsewhere in the body (*21*). It is generally concluded that the reaction is mediated by a chemical factor which is organ specific, since other types of organ are not affected, and which circulates systemically in the blood so that sites distant to the place of damage are affected. These

two assumptions are incorporated in most general theories of growth control, irrespective of whether the chemical factor is believed to be a metabolic product of the organ (*21*), a chalone (*22*), a wound hormone (*23*), or some immunological product (*24*).

In the case of the chalone hypothesis, the postulated reason for compensatory growth is that, whereas in the intact animal the organ is under the influence not only of locally produced chalone, but also of chalone from homologous organs reaching it by its afferent blood supply, following extirpation of the homologous organ the latter source of supply is decreased and the total concentration of chalone within the remaining organ thus decreases. There is, however, another factor which could cause a local reduction in chalone concentration without the need to postulate a change in the afferent supply of chalone. If liver lobes are dissected from a partially hepatectomized rat, they rapidly become very pale, due to blood loss; in contrast, liver taken from intact animals retains its typical blood-engorged color. Vascular changes evidently occur in the remaining lobes after partial hepatectomy, resulting in a much more rapid efferent blood drainage.

The effect of this might be to remove chalones more quickly from the organ, and it could be postulated that it was a decrease in the locally diffusible chalone pool rather than in the systemically circulating pool which constituted the deficiency message leading to the growth stimulus. If growth is stimulated by local rather than systemic changes, then it is possible to argue that the mechanism could function without the regulatory growth-inhibiting substances being strictly organ specific in their chemistry. This argument is somewhat tenuous and depends on whether an increased rate of growth-inhibitor clearance from one particular organ would increase the amount present in the circulation to the extent that growth processes in other organs would be inhibited. Dilution alone would diminish this effect, and the build-up of an effective systemic pool would also depend on the inhibitor being relatively stable. Steroid hormones, which are tissue-specific growth-control substances for some endocrine-dependent organs, have a very rapid clearance rate: The half-life of adrenocorticotropic hormone in rat blood is 5.5 minutes (*25*) while that of estrogen may be as low as 2 minutes (*26*). It is possible that substances which regulate compensatory growth in other organs may be similarly short-lived.

A compensatory cell-division response to tissue damage is found in a large number of organs. If changes in the rate of blood flow mediate in this reaction, they must be equally widespread. This appears to be the case. In many tissues, local damage is typically followed by an inflammatory response which entails an increased rate of blood flow (*27*). Partial extirpation of liver (60–70%) in rats approximately doubles the rate of blood flow per unit weight of remaining liver, though this returns to normal after 5 days (*28*). The mitotic response in the regenerating liver is dependent on an efficient vascular supply (*29*) while the fact that the volume of blood delivered appears to be the determining factor, rather than whether the blood is of portal or caval origin, suggests that the response is due to the flow rate per se rather than to some hypothetical control factor present in the afferent blood supply (*30*).

The relationship between blood flow and mitotic response, well documented in the liver (*31*), has received less attention in other organs, although it is known that collapse of pulmonary tissue is followed by the diversion of blood away from the functionless areas to other parts of the lung (*32*) which then show an increased mitotic rate (*12*). However, the rate of blood flow in most organs is subject to considerable variation under the control of hormonal and neural mechanisms (*33*). A case in point is the kidneys which normally receive one-third of the cardiac output of blood but in which blood flow can be decreased almost to zero by the action of the sympathetic nerve supply (*32*). Although unilateral nephrectomy produces no rapid increase in the overall rate of blood flow through the contralateral kidney (*34, 35*), it is known that experimental treatment can produce marked local changes in blood flow through the action of bypass systems within the kidney (*36*).

The possible role of blood flow in controlling regeneration by increasing the rate of clearance of control factors therefore merits investigation.

As experimental models it would be useful to have situations in which a mitotic response accompanies increased blood flow in the absence of actual tissue loss. The example of the collapsed lung has already been cited, and tissue grafts may also provide suitable situations. In skin grafts (*37, 38*) and in subcutaneous implants of whole embryos (*39*), both of which become highly vascularized, large increases in the rates of cell division have been observed.

A large body of evidence concerning growth control factors comes from experiments in which serum fractions or subcellular fractions are added to living systems with a view to modifying the rate of cell proliferation. By assuming the need for tissue specificity in such effects, it is possible that relevant information could be disregarded, since, as already discussed, the clearance-rate hypothesis may not require strict chemical specificity. Whether control by nonspecific factors is feasible could be decided from cybernetic principles using models which incorporated functions for the rate of production and breakdown of control factors, the rate of blood flow, and the degree to which soluble factors were diluted in the circulation.

MITOTIC REGULATION AND THE CELL CYCLE

Following administration of many growth control substances the effect on mitosis is so rapid as to indicate a direct action on the movement of cells from G_2 to M; mediation by some previous point in the cell cycle would involve a delay. In the case of chalones, direct action on G_2 appears to occur in the epidermis (*40*), lung (*41*), and pronephros (*42*). However, following tissue damage, stimulation of DNA synthesis (movement from G_1 to S) occurs a few hours before mitotic stimulation (*1*), and it is therefore difficult to envisage a growth control model based solely on control at the level of G_2 to M. It has been suggested (*43*) that control of the cell division rate is through some critical process preceding DNA synthesis, after which division proceeds without further external control. But this again appears to be an oversimplification.

Following explantation of a number of tissues in organ culture there is a large increase in the rate of entry into DNA synthesis, which may come to be several times higher than the rate of mitosis itself (*44*). In the lung this leads eventually to an accumulation of cells in G_2 (*45*). The phenomenon is not due to temporary synchronization of the cell cycle or to decreased tissue viability, and it therefore demonstrates that the rate of mitosis is not determined directly by the rate of DNA synthesis. Some coordinating mechanism must exist in the intact tissue in vivo. Whether chalones, or other regulatory factors such as somatotropic hormones, can themselves exert such a coordinated control is not known.

While it has been shown that the epidermal chalone may act both on mitosis (*46*) and on DNA synthesis (*47*), similar information for other systems is not so readily available. This is an important line of research because the validity of chalones as control factors of biological significance will rest on their ability to explain the intricacies of growth control phenomena observed in vivo. In this context, the possible function of stress hormones is of interest, since they appear to be important intermediates in chalone action (*40, 48*). Adrenaline depresses mitosis in a number of tissues but in many cases has no direct action on DNA synthesis (*49*). Conversely, hydrocortisone, which inhibits the movement of cells from G_1 into S (*50, 51*), may have no direct action on mitosis (*18, 49*). The differential action of two control substances is one way in which the different parts of the cell cycle could be coordinated.

CONCLUSIONS

Much of the classic work on chalones involves the action of crude or partially purified tissue extracts, and in many cases the effects observed depend on time-consuming methods of experimental analysis which have discouraged other workers from attempting to verify the results obtained. There is good argument for attempting the chemical purification of chalones by using biological systems which lend themselves to rapid and straightforward analysis. It should nevertheless be remembered that there are many aspects of cell proliferation which cannot yet be explained in terms of our limited knowledge of

how chalones act on the cell cycle and that the growth processes observed in embryonic or regenerating organs depend upon a complex control system which involves morphogenetic interactions between tissues in addition to the homeostatic regulation of cell division.

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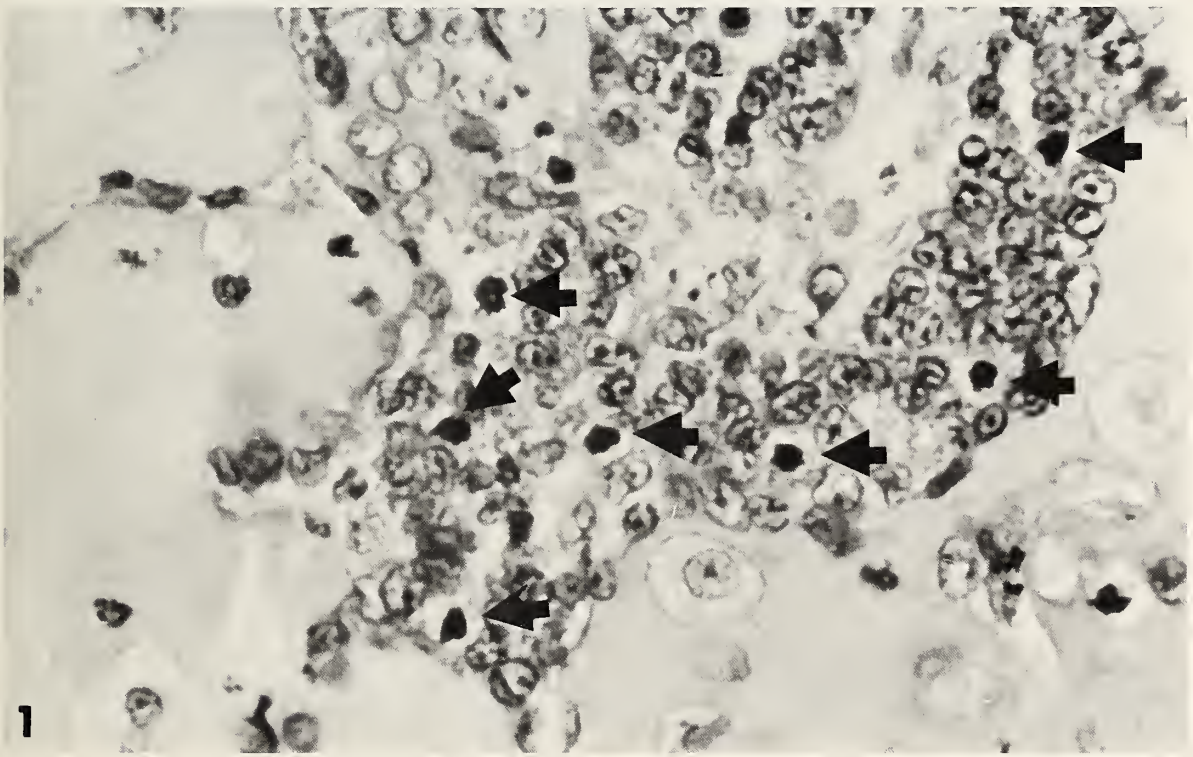

Figure 1.—Right lung after removal of left lung, showing stimulation of mitosis (*arrows*) in bronchial epithelium. Hematoxylin and eosin. × 1,000

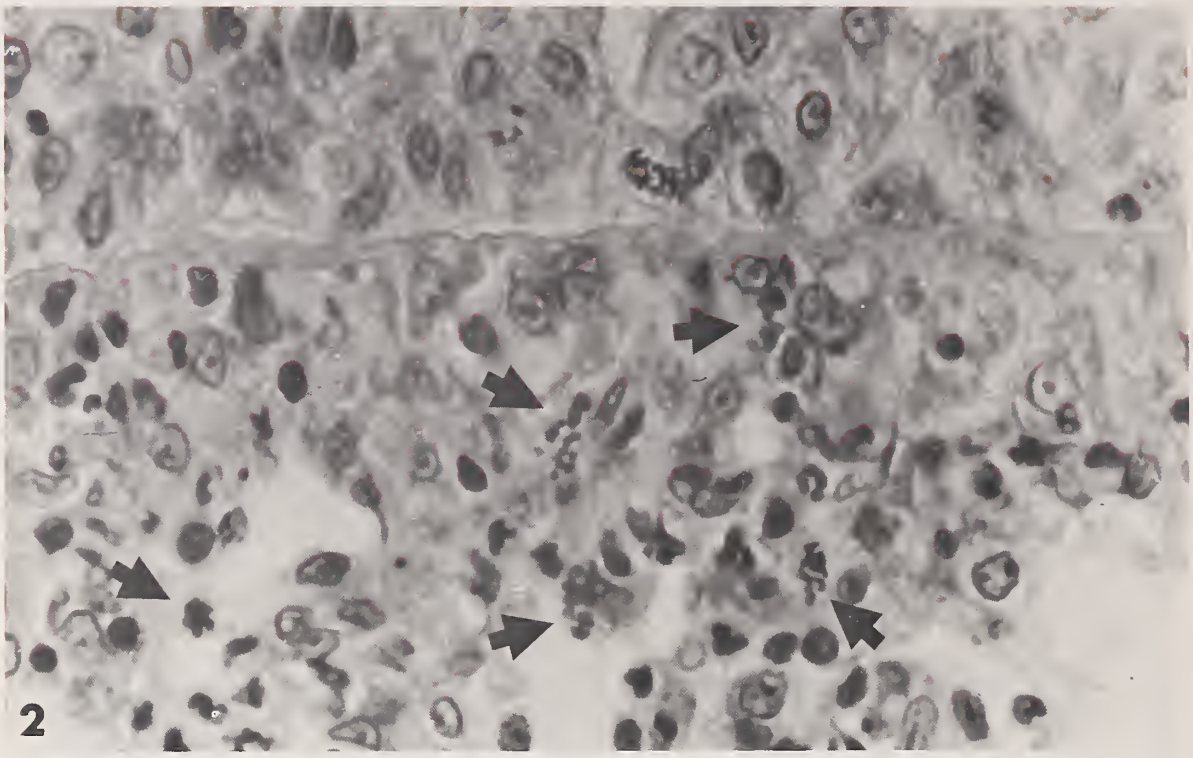

FIGURE 2.—Right lung after removal of left lung, showing dense subpleural layer of interstitial cells containing abundant mitotic figures (*arrows*). Hematoxylin and eosin. × 1,000

Experimental Approach to the Epidermal Chalone [1]

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SUMMARY—The experimental action of epidermal chalone on cell division in normal epithelia, both in vitro and in vivo, and in experimental pathological conditions is reviewed. A short history of the discovery of the epidermal chalone is included together with an account of the various extraction and isolation procedures used. The physicochemical and biological properties and the interaction with stress hormones are considered. Also reviewed is the literature on the action of the epidermal chalone on the cell cycle and its production by the cells of the epidermis. The effects of the chalone on cell division after experimental surgical wounding of the skin, after application of chemical carcinogens, and in transplantable tumors are included. It is apparent that there is considerable discrepancy between results from in vitro experiments and from those performed in vivo. Also, the various extracts from different laboratories are not identical and even may not be chalones. There seems to be considerable emphasis placed on negative results, which may lead to unsatisfactory conclusions. It is concluded here that there is an outstanding need for a quick, reliable, discriminative test which does not rely on negative results. The present lack of large quantities of pure chalone is regrettable; in addition, there is still not enough knowledge of the relevant biology and kinetics of epidermal cells. Only a complete knowledge of the chemical composition and structure would resolve the possibility of the name "epidermal chalone" being applied to totally different substances.—Natl Cancer Inst Monogr 38: 37–45, 1973.

THERE ARE now so many reviews on chalones [e.g., (*1–10*)] that, if it were not for the sudden increase in interest in chalones during the last 2 years, there would be grave danger that the reviews would outnumber the people working on chalones. It is noticeable, however, that theoretical considerations tend to predominate and there is now a need to attempt to summarize some of the published experimental evidence which in the past formed the basis for the theory and which now forms the substance for challenging, confirming, modifying, or rejecting the present chalone concept. This, after all, is the reason for this conference. It is the purpose of this review to present historically the experimental evidence from the point of view of the epidermal chalone.

INTRODUCING THE EPIDERMAL CHALONE

The experiments with differential wounding, published by Bullough and Laurence in 1960 (*11, 12*), were the first positive experimental attempt (although the evidence is only circumstantial) to show that epidermal mitotic activity is

[1] Presented at the First Symposium of the International Chalone Conference, Brook Lodge, Augusta, Mich., June 5–7, 1972.

[2] I should like to take this opportunity to thank all the technicians who have helped to make this work on epidermal chalone in London possible. If it were not for their devoted assistance in this laborious work, it is possible that this conference would not be taking place or, at the most, would be postponed for a few years. To name a few who gave assistance for many years, I thank Maureen and Roy Thompson, Hanna Deol, Sandra Fayers-Jessop, Sodja Ahulu, Shirley Knibb, Paul Taylor, Tony Long, Elizabeth Bunn, and Anna Bobinska. I also thank the technicians at N.V. Organon who made available the large quantities of skin extract.

regulated by an inhibitor produced by the tissue itself. These experiments [subsequently confirmed by Finegold (*13*)] have been quoted so often [e.g., (*8, 14–16*)] that they need not be reviewed here. Independently, at that time Iversen in Oslo was determining the mitotic response to application of 3-methylcholanthrene to the skin of hairless mice (*17*) and Tsanev in Sophia was studying the change in RNA content of epidermal cells after they had been damaged by pressure (*18*). The subsequent analyses with the use of computers by both led them to conclude that a single substance, acting by means of a negative-feedback system, was necessary to control the homeostasis of normal epidermis (*19–22*). Thus, independently, in three laboratories in three different countries, the experimental approach to an understanding of "epidermopoiesis" by a negative-feedback mechanism became a way of life. In September 1959 the first attempts were made in London to extract this inhibitor.

In 1959 the problem was simply to find this unknown substance of unknown composition, the source and action of which were only indirectly indicated and for which there was no known assay system. At that time, autoradiography was in its infancy and the knowledge of the premitotic phases of the cell cycle was ill defined. Liquid scintillation counting was not available, and some sophisticated biochemical techniques now in regular use were yet to be conceived.

It is always difficult to make investigations on a mitotic inhibitor; in an in vitro system, for example, a change in osmotic pressure of the solution or a trace of toxic substance can depress mitotic activity. Difficulties also exist in an in vivo system (*24*). For example, the mere fact that animals are awake can depress the mitotic activity of mouse epidermis (*23–26*). Therefore, any unusual disturbance in the animal house can cause misinterpretation of results. Bullough and Johnson had already established a technique for the study of mouse ear epidermis in vitro (*27*), and at that time work was in progress on the effect of stress and stress hormones using this in vitro system (*24*). It was decided to exploit it (*28*) since, in in vitro work, less material is needed and it is easier to study the effect of a single substance.

In the first instance, the ears of the mice were split and then ground by hand in a glass macerator. The resulting liquid was tested on small pieces of mouse ear, incubated at 38° C for 4 hours with Colcemid in phosphate-buffered saline with 0.02M glucose and oxygen as the gas phase. Subsequently, extracts were made by plucking the hair from mice and scraping the skin to remove the epidermis. These scrapings were then macerated in a Potter-Elvehjem homogenizer and centrifuged at 3000 rpm. For years this was the standard laborious method of obtaining epidermal extract, and the standard method of testing was an extension of this in vitro technique. The next few years of intensive experimental work on this inhibitor, called the "epidermal chalone" by Bullough in 1962 (*29*), showed that it was only active when adrenaline (endogenous or exogenous) was present (*30*).

It was necessary to establish a discriminative test, so that the inhibition caused by the chalone could be distinguished from that caused by a general toxic substance. Luckily, in the in vitro system the inhibition due to chalone lasts 5 hours and full recovery is made by 9 hours. Unfortunately, this does not eliminate the same effect that is produced when the inhibitory power of a solution is lost as for example with adrenaline (*30, 31*). When new ears were placed in extracts which had been incubated for even up to 12 hours previously at 38° C, the chalone still inhibited the mitotic activity in the subsequent 4 hours (*32*). This showed that during the incubation period the ears had lost their ability to react to epidermal chalone, but the inhibitory power of the extract remained. This resulted in the development of the discriminative test for epidermal chalone using the "adrenaline wash" technique (*30*). This technique was used extensively by Bullough, Hewett, and Laurence (*33*), Hondius Boldingh and Laurence (*34*), and, subsequently, by Marrs and Voorhees (*35, 36*) in their attempts to isolate the pure substance, and also for the detection of the epidermal chalone in other epithelial tissues (*30, 37–39*) and in tumors of epithelial origin (*40–42*). The epidermal chalone was said to be present in an extract only

when there was a mitotic depression at the end of 5 hours of incubation and after 9 hours of incubation with the "adrenaline wash" inserted at 5 hours as well as no depression after 9 hours of incubation in the extract alone.

While this work was in progress in London, Iversen was continuing his studies of epithelial cell kinetics (*43*). Experiments with epidermal chalone began when he was joined by Elgjo. Their approach used an in vivo assay relying on the hypothesis that the inhibition of the chalone was tissue specific. Their standard assay involved the injection of extracts of skin into hairless mice; the response was an inhibition of mitosis in the interfollicular epidermis of the back skin. Extracts of liver prepared by the same method had little or no inhibitory action (*44*).

In Helsinki in 1965, tissue-specific mitotic inhibitors, with the name "chalone," made their first conference appearance (*45*). The reception was one of total disbelief or skepticism. At that time the only other chalone being studied was that of the granulocytes, by Rytömaa and Kiviniemi (*46*). To work with chalones it is necessary to have a considerable amount of determination, and it is noticeable that, at the first Chalone Club meeting in Oslo in 1968, 9 years after the first epidermal extracts had been made, there were only 12 people who could possibly participate and only 3 chalones were represented. By then, the melanocyte chalone had been introduced (*47, 48*). The one outstanding result of this meeting, however, was the formulation of the biological properties of chalones by Rytömaa (*10*) as follows:

"The chalones inhibit mitotic activity both in vitro and in vivo; their action is reversible, tissue specific but not species specific; and some chalones require stress hormones as cofactors."

The distribution of epidermal chalone and the application of the chalone theory to wounded tissue (*49, 50*) and to tumors (*40–42, 51*) were consequent investigations natural to biologists and pathologists. Attempts have been made to identify the active principle (*34, 36*) and to ascertain the biological action as well as to understand the mechanism of this action (*52–55*). The following account is a résumé of the experimental findings.

ISOLATION OF EPIDERMAL CHALONE FROM PIG SKIN

Preliminary attempts to isolate the epidermal chalone using the now standard "adrenaline wash" technique for identification were made by Bullough, Hewett, and Laurence (*33*) but it was obvious that, if large quantities were going to be needed, scraping mouse skin was hardly practicable. The help of N.V. Organon, involving in particular Dr. W. Hondius Boldingh, was sought. The following account is a short summary of this work, most of which has been published (*34*), using pig skin as a starting material.

From experiments performed between 1963 and 1966 it was found that the best procedure for large-scale production of epidermal chalone was to obtain skin powder from commercial pig rind, to lyophilize, grind to a fine powder, and extract with ice-cold distilled water in nitrogen at 0–4° C, and then to centrifuge. This crude water extract was fractionated with ethanol, the active principle being precipitated when the alcohol concentration was increased from 71 to 80%. This concentrated the active fraction 25 times. Column electrophoresis of this ethanol fraction was carried out at pH 3 in 0.1M acetic acid; this concentrated the active fraction by a factor of 125. Finally, exhaustive dialysis of the active electrophoretic fraction gave a 2,000-times purification from the crude water extract. Pepsin extraction, although giving a good yield, and acetic acid extraction produced viscous and gel-like suspensions which were difficult to handle.

Attempts to separate the active fraction on Sephadex columns were not ideal because on G-75 some activity was spread throughout. Similar results were obtained with ion-exchange chromatography using diethylaminoethyl Sephadex and carboxymethyl cellulose. Even when the pH was raised to 5.2 (instead of pH 3) for the column electrophoresis used above, there was no definite condensation of activity. It was unfortunate that, for example, molecular sieving or isoelectric focusing was not available. However, it is hoped that these results will form a basis for further work. Because of the difficulties of large-scale column electrophoresis and dialysis, the ethanol fraction was used for most biological ex-

periments while the most purified fraction was used for physical and chemical analyses.

SOME PHYSICOCHEMICAL PROPERTIES OF EPIDERMAL CHALONE OBTAINED FROM PIG SKIN

The elementary composition, amino acid, and carbohydrate analyses together with the effects of proteolytic enzymes indicate that the chalone is a simple protein or glycoprotein. In its purest form it was almost homogeneous as shown by immunochemical diffusion, gel electrophoresis, and ultracentrifugation. It has a molecular weight between 30,000 and 40,000 as determined on Sephadex columns and by ultracentrifugation. The isoelectric point as determined by column electrophoresis was between 5.2 and 6.0. The ultraviolet spectrum showed a gradually decreasing extinction with increasing wavelength between 220 and 300 nm. The specific optical rotation was about $-80°$ (nonhelical).

The chalone is degraded by trypsin but not pepsin, it is stable at low pH and is degraded completely at pH 9, and it is stable at room temperature in sterile solution for 1 week, the activity being subsequently lost by 8 weeks. The chalone is active for at least 10 hours at 38° C but is inactivated by heating at 100° C for 10 minutes. Finally, it has been stored for at least 6 years in lyophilized form.

DISTRIBUTION OF EPIDERMAL CHALONE

Using the in vitro "adrenaline wash" technique and the epidermis of mouse ears as the assay system, it has been shown that epidermal chalone is present in the skin of various mammals and the codfish (*56*), rabbit and mouse lens (*38, 39*), pig palate and gingiva and human gingiva (*37*), pig esophagus (*57*) and lung (*58*), and human urine (*58*). It has been isolated from three different tumors of epidermal origin (*40–42*) and from mouse and human lung tumors (*58*). It has not been found in pig liver or striped muscle (*58*), mouse brain or rectum (*30*), sheep lymphocytes (*59*), or bovine granulocytes (*60*); it has not been found in mouse lymphoma (*59*) or in mouse and hamster melanomas (*48*).

Thus it appears that the epidermal chalone may be extracted from organs which contain epithelial tissue. This poses the problem as to whether the implications in the name "epidermal chalone" are correct. This aspect is considered in a later paper of this conference (*57*). However, it is worth noting here that the epidermal chalone extracted from pig skin is active in vitro on human epidermis (*61*), on HeLa cells (*8*), and on rabbit lens epithelium (*38*).

BIOLOGICAL PROPERTIES OF EPIDERMAL CHALONE

The discriminative test has shown that the reaction to the epidermal chalone is reversible. Species-nonspecificity has been demonstrated but tissue-specificity has been questioned, since the epidermal chalone has been found in many different epithelia.

Tissue-specificity is always very difficult to demonstrate because it is necessary to prove that the action is on the epidermis alone and not on other tissues. To prove a negative statement is virtually impossible, but an attempt has been made by using the sebaceous glands as the test object in the same pieces of ear that were used for the epidermal study (*62*). It was shown that the sebaceous gland chalone is present in the ethanol fraction of pig skin but not in the more purified dialyzed fraction. No action on mitoses in sebaceous glands was found with epidermal chalone extracted from other epithelia—e.g., extracts of oral epithelia (*62*), esophagus or lung (*58*), or from the skin of the codfish (*62*) where no sebaceous glands occur. Equally, activity has not been found in extracts of the three epidermal tumors studied (*40–42*). The epidermal chalone has no effect on the mitoses of the eccrine sweat duct (*63*) or on mitoses in hamster and mouse melanomas (*48*) or mouse lymphoma (*59*). It must be remembered, however, that both sebaceous glands and the eccrine sweat ducts are formed embryologically from epidermis. This problem is considered in more detail elsewhere (*57*).

The Oslo school has extracted liver by a method similar to that used for their epidermal chalone, and both extracts have been tested in vivo on mouse epidermis. They find no, or only

slight, effect with liver extract compared to a marked depression with extract of skin (*8, 51, 64*). Unfortunately, this does not eliminate an effect which could be due to dilution of chalone.

EPIDERMAL CHALONE AND STRESS HORMONES

Adrenaline has played a major role in the identification of the epidermal chalone, and it has been suggested that adrenaline acts in some way as a cofactor (*30*). The action of the two substances combined is never additive (*65*), and yet adrenaline is necessary for chalone action. In a complicated series of experiments, Bullough and Laurence have shown that glucocorticoids, in this instance hydrocortisone, may be equally important in chalone action (*65*). Apparently hydrocortisone prolongs the time that the mitotic depression lasts with chalone. They concluded ". . . that the antimitotic power of the chalone is augmented by adrenaline while . . . the role of glucocorticoid hormone may be to sustain the adrenaline effect. . . . The actual manner of interaction of chalone, adrenaline and hydrocortisone is quite unknown."

The whole question of interaction of these three substances has been reexamined as a result of a comparative study in vivo on oral epithelium and ear epidermis (*57, 66*). In a short review on this subject, Laurence et al. (*67*) presented evidence to show that the action of the stress hormones is only indirectly on the chalone itself. It appears that the stress hormones act on a substance which is present in the dermis. This substance normally negates the chalone action, allowing mitosis to occur, and has been referred to as a "chalone antagonist."

ACTION OF EPIDERMAL CHALONE ON CELL CYCLE

Since the epidermal chalone has been studied mainly by virtue of its depression of visible mitosis and, since this effect is seen within the first hour (*30, 66*), it may be inferred that the cells are blocked in the G_2 phase of the cell cycle (*68*). This observation has also been made by Schilling et al. (*69*) working with human primary foreskin in vitro. In addition, Bullough and Laurence (*25, 70*) showed that the duration of visible mitosis is lengthened, both in vivo and in vitro, by the administration of epidermal chalone. However, Iversen and Elgjo (*64*) found no change in the mitotic duration in the hairless mice with their extracts of skin.

Work on the effect of epidermal chalone on DNA synthesis in the epidermis is still in its infancy, and results so far appear inconclusive. Baden and Sviokla (*71*), working with rat skin in vitro, found no change in the uptake of ^{3}H-thymidine over a 3-hour incubation with skin extract, while Hall (*72*), working with organ culture of hamster cheek pouch, found an inhibition of cells entering S phase. A similar result was found by Marks et al. (*73*), Dahl and Shuster (*74*), and Delescluse et al. (*75*) working with various culture systems. Hennings et al. (*76*) have shown that there is a lag of 9–12 hours before the depression is apparent. These authors and Frankfurt (*50*) showed the necessity for several injections of epidermal chalone over a 12-hour period in order to demonstrate a blockage of cells entering S phase.

At this point one wonders if everyone is talking about the same substance or if there is more than one chalone-like inhibitor, or even whether the effect is due to chalone at all. There also seems to be a very clear indication that the in vitro systems in no way reflect the conditions in the animal. Elgjo (*68*), in a long study over the 24 hours following the injection of extract, found no increase in mitosis after the 4-hour blockage at G_2, indicating that some cells never passed through visible mitosis. However, with the diurnal rhythm in mitotic activity playing such a decisive role in the epidermis, it is difficult to interpret this experiment solely on the effect of chalone action. Also, when tested in vivo, his extracts are only stable for 30 minutes (*77*) but, in vitro with the "adrenaline wash" technique, they have been found to be active over the 9-hour period (*32*).

Elgjo et al. (*52, 53, 78*) have proposed that one inhibitory substance affects cells in G_1 whereas another inhibits cells in G_2. This concept is supported by Marks (*79*) who has shown that there is a tissue-specific, heat-stable inhibitor in the

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extracts of pig skin used by Bullough, Hondius Boldingh, and Laurence, which has an even higher molecular weight than the epidermal chalone that is known to act on G_2. This inhibitor acts in late G_1 of the cell cycle. It is interesting that this G_1 inhibitor is not affected by excessive digestion with pancreatic ribonuclease, trypsin, or pronase.

One can only conclude from all these results that not enough knowledge exists on the biology of epidermis, on the specific actions of the extracts used, on their chemical composition, or on their biological action. Apparently, a good standard test system for epidermal chalone has yet to be found.

In an attempt to resolve this problem, Powell et al. (*55*) have exploited the fact that adrenaline is necessary before there is any response to epidermal chalone. Their work with cyclic AMP [the role that was primarily suggested by Iversen (*8*) and shown to be a possible mediator by Brønstad et al. (*54*)] will lead, one hopes, to a solution of this problem of a good test system. However, as yet, no positive proof has been established of the role played by cyclic AMP in chalone action.

PRODUCTION OF EPIDERMAL CHALONE

There is very little doubt that the epidermal cells produce epidermal chalone, but it is not yet known whether it is produced by the basal cells or the cells of the spinosal or granulosal regions of the epidermis. Elgjo and his colleagues have made a concerted effort to solve this problem by trypsinizing the epidermis of hairless mice and extracting the cells of the basal layer and separately those of the differentiated layers. They conclude that the basal cells produce a fraction which inhibits G_2 and not G_1 (*53*) and that the outer spinosal cells produce a fraction active on G_1 and not on G_2 (*78*). It should be recalled here that Hondius Boldingh and Laurence (*34*) showed that the epidermal chalone that acts at G_2 is inactivated by trypsin, while Marks (*79*) has shown that the inhibitor acting at G_1 is trypsin resistant.

EPIDERMAL CHALONE AND EXPERIMENTAL PATHOLOGICAL CONDITIONS

Having accepted that chalones do exist and that the experiments with differential wounding (*11*) indicate that there is a loss of this inhibitor from the wounded epithelium, it was natural that the effect of epidermal chalone on wounded epidermis should be studied. An in vitro technique for the study of small wounds already existed (*80*). This was elaborated as a result of the available knowledge which indicates that chalone action is enhanced by the addition of adrenaline and hydrocortisone (*49*). It was surprising that epidermal chalone by itself gave only a small mitotic depression. If there was less chalone present in the wounded epidermis, one would expect a greater response. It was also significant that the combination of adrenaline and hydrocortisone gave the same depression as when all three substances were added. This indicates that there is a greater loss of chalone from the wounded cells and that production of chalone could still be high. Whether added epidermal chalone would delay the onset of increased mitotic activity in wounds has not been determined, but Frankfurt (*50*) has studied the effect of epidermal chalone on the increase in mitosis during experimental hyperplasia in vivo. He showed that the epidermal chalone inhibited the increase in cell number and that the effect persisted for 14 hours after the cessation of the epidermal chalone treatment.

The study of tumors presents many problems. The proliferative zone is not so readily definable as in epithelia and so the technique is much more subjective. Of the three epidermal tumors studied, one was nonkeratinizing (*40*), while the other two were keratinizing (*41, 42, 51*). All produced the epidermal chalone. All reacted to the epidermal chalone, in some circumstances, by a mitotic depression. However, no dramatic cure for epithelial carcinoma has been demonstrated yet (*51*). To attempt this with some purpose, enormous quantities of pure chalone are necessary and a greater knowledge of the action of adrenaline and hydrocortisone, at least, is needed.

CONCLUSION

This outline of the experimental findings on the epidermal chalone tends to give a confused image. However, certain questions need to be re-

solved before a clear understanding of chalones and their importance can be achieved. The outstanding question remains: Is everyone examining the same substance?

The indications are that they are not. It is not even certain that all substances described as epidermal chalone are chalones at all. This doubt stems from our lack of knowledge in two fields: 1) the biology of the epidermis and 2) the chemical composition of the epidermal chalone. Until a good test system involving the real epidermal chalone is known and large quantities of the pure substance are made available, the many problems existing at present and, no doubt, to be formulated in the future will remain unresolved. The need for greater discrimination and clarity is obvious.

However, the 1970's saw a change in attitude to chalones and particularly the epidermal chalone. Not only is research on epidermal chalones (or what is reported to be epidermal chalone) being performed in England and Norway but also it now is going on in laboratories in France, Germany, Switzerland, America, Russia, and Japan. Whereas in 1965 only 2 chalone systems were known to exist, there are now 12, and active research on chalones is taking place in at least 13 different countries. It is a pleasure to be joined by so many instead of convincing so few.

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Role of Cyclic AMP in the Control of Epidermal Cell Growth and Differentiation [1, 2]

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SUMMARY—A model of epidermal growth control is presented, which seems to account for many observed facts, both old and new. It gives each epidermal cell a sphere of influence, with the ability to adjust its own proliferative capacity and that of its neighbors via the second messenger and morphogen, cyclic AMP. In this regard the intracellular levels of cyclic AMP may not be under primary hormonal control but may be controlled by a specific local substance previously called a "chalone." Until this supposedly tissue-specific, species-nonspecific molecule or series of molecules have been chemically purified from histologically pure epidermis, we suggest that the term "chalone" be reserved for the overall concept of epidermal autoregulation. In this way, modern chalone research can be integrated into the mainstream of the massive amount of investigation which is under way on the nature of growth control in cancer. In this presentation we discuss what in our view is a neglected but nonetheless excellent model of disturbed growth control—i.e., psoriasis. Psoriasis shares many features with cancer but psoriasis in itself never becomes malignant. An understanding of why psoriasis, a disease afflicting 6 million Americans, is not cancer and why cancer is not psoriasis would in all probability bring us much closer to an understanding of both disorders.—Natl Cancer Inst Monogr 38: 47–59, 1973.

THE DELICATE balance between proliferation and differentiation, which is characteristic of normal keratinization, is probably maintained by multiple inhibitory and stimulatory influences of epidermal, dermal, and systemic origin as well as by topological factors in the epidermis and dermis. Our attempts to identify a "key control mechanism" in this seemingly unapproachable array of regulatory factors have been guided by several biological observations made in certain squamous cell carcinomas of epidermis (*1*), in wounded epidermis (*2*) and other epithelia, and in the pathologically accelerated epidermal proliferation and differentiation of psoriasis (*3, 4*). In superficially damaged epidermis (*5*), cornea (*5*), and esophogeal (*6*) and gallbladder epithelia (*7*), the accumulation of glycogen, the DNA synthesis, and the mitotic activity occur within several hours of injury. Furthermore, these events occur in the immediate vicinity of the injury and not throughout the injured tissue.

In the multifactorial genetic disease, psoriasis (*8*), a disorder afflicting approximately 6 million Americans, a reaction resembling wound healing

[1] Presented at the First Symposium of the International Chalone Conference, Brook Lodge, Augusta, Mich., June 5–7, 1972.

[2] Supported in part by Public Health Service research grants NIH–NCI–E–72–3216 from the National Cancer Institute and AM15740–01 from the National Institute of Arthritis and Metabolic Diseases and by Public Health Service general research support grant RR05383–09 from the Division of Research Resources. Also supported by institutional research grant IN–40J from the American Cancer Society, Inc.

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is seen. In the lesion the epidermal cell cycle is accelerated by a factor of approximately 12 (each cell dividing every 1½ days rather than the normal of 19 days) (*9–11*), differentiation is incomplete (*12*), and glycogen levels are increased in the hyperplastic epithelium (*13, 14*). Although it has been assumed that the incomplete differentiation of psoriatic epidermis is secondary to the rapid cell cycle, the possibility that the rapid proliferation is secondary to the defective epidermal differentiation has by no means been ruled out. The lesions are discrete, hyperplastic patches which appear spontaneously or after wounding, last several weeks to months, and frequently disappear spontaneously only to reappear elsewhere or in the same spot.

In normal epidermis which has been damaged, Lobitz et al. (*2*) found increased glycogen within several hours, whereas mitosis occurred after 48 hours. Pinkus (*15*) showed that very superficial epidermal damage (that produced by 4 cellophane tape strippings) without associated histologic evidence of dermal change results in epidermal proliferation below the superficial injury. Although no histologic evidence of dermal injury was seen, epidermal mitotic stimulators could have been liberated from the dermis. However, considerable evidence exists to indicate that neither inflammation alone nor dermal injury alone results in epidermal proliferation (*16*). It appears that the epidermis itself must be damaged (*15, 16*). If such damage were to interrupt a control mechanism which operated by chronic stimulation of epidermal proliferation, the epidermis clearly would not regenerate.

The available evidence suggests that the balance between normal epidermal proliferation and differentiation is maintained by a mechanism which promotes differentiation out of the G_1 phase of the cell cycle, thereby indirectly restricting the number of cells available to enter the DNA synthetic phase of the cell cycle (*17*). Alternatively, such a mechanism could operate directly by inhibiting the G_1 phase of the cycle with little direct relevance to differentiation or having direct relevance by giving the slowed G_1 cells "the opportunity to decide" to differentiate. We prefer the former mechanism of stimulated differentiation, since the tissue specificity of any such mechanism would seem to lie in the differentiated state of the tissue (*18*). The reason for this preference is that cellular proliferation per se is a highly fundamental and thus possibly nonspecific property of those cells, throughout the body, whose cell cycle reproduction genes are still open for transcription.

In this scheme, any injury would disturb the differentiated state of the cell by damaging the molecular mechanism responsible for its maintenance. In the absence of a normal epidermal differentiation maintenance or stimulatory mechanism, the epidermal basal cell would have little else to do other than continue to divide, since it is not yet in a geographical or molecular position to cornify and die. Since certain of the damaged differentiated cells may die before cornification, such a mechanism would provide replacement cells which would then differentiate because their molecular mechanism for the promotion of differentiation would be intact. As such differentiation of newly formed cells occurs, cellular proliferation would thereby be limited. This concept is similar to the template-antitemplate model of Weiss and Kavanau (*19*), Bullough's chalone concept (*18*), and Iversen and Bjerknes's theory (*20*) of a cybernetic system in the epidermis utilizing negative feedback for the control and stabilization of epidermal growth.

Although these growth control theories account for the homeostatic equilibrium maintained between growth and differentiation, they do not include an explanation for the appearance of glycogen in regenerating epidermis before the onset of cell division (*2*). McMinn states that ". . . probably the most striking histochemical change in epidermis after wounding is the accumulation of glycogen" (*5*). In this presentation we will review the concept of the chalones, summarize our data on the role of cyclic AMP in proliferation and differentiation of normal and psoriatic epidermis (*3, 4, 21–32*), and discuss the work of other investigators on the role of cyclic AMP in the control of cellular growth. Then, with due respect for the fact that most theories are wrong and that a subject as complex as growth and differentiation may not be ready for another theory at this time, we will offer a theory for epidermal growth control which might have rele-

vance to other continuous cell renewal systems.

This theory will embrace the chalone concept, account for the glycogen in proliferating epidermis, and include the considerable body of evidence implicating cyclic AMP in growth control. Our apologia for yet another theory is twofold: 1) It appears to be testable in its entirety with available technology and thus can be established, revised, or discarded within several years; 2) to be without a testable theory which seems to encompass established biological observations summarily relegates one to being an investigator rather than a discoverer, a position in which no-one wishes to be.

EPINEPHRINE-CHALONE SYSTEM

Since it was the early work of Bullough and Laurence on what was called the "chalone-adrenaline complex" that led to our current studies on the role of cyclic AMP in epidermal proliferation, we wish to summarize what was known of this complex in 1964 (*33*). Later in the presentation we will attempt to reinterpret Bullough and Laurence's observations in light of our recent work on cyclic AMP and then to unite the chalone concept and the cyclic AMP concept into a single theory.

In 1962 Bullough (*18*) suggested that epidermis contained a growth-controlling factor which he called a "chalone." Bullough's notion was that such a substance promoted differentiation, thereby limiting the number of cells available to pass through the cell cycle. In 1964 Bullough and Laurence (*33*) further suggested that the known reversible G_2 epidermal mitotic inhibitor, epinephrine, acted in concert with a locally produced chalone. It was suggested that neither epinephrine nor chalone alone had any growth regulatory activity. The work at that time (*33*) was based on a primarily G_2 cell cycle assay, although the proposed mechanism of epinephrine-chalone action seemed to require that epidermal differentiation be promoted out of the G_1 phase of the cell cycle (*18*). This is true of most cells and is true of epidermis as well, since Bullough has shown that epidermal cells above the basal layer have a 2N DNA complement rather than a 4N (the DNA content of G_2 cells) as determined by cytospectrophotometry (*34*). This discrepancy of a G_1 control mechanism based on data generated in a primarily G_2 assay and a lack of purified chalone or chalones has given us conceptual difficulties. Nonetheless, we have been intrigued by the chalone concept and believe that it is a fundamentally sound theory.

In vitro (*33*), the following is known of the epinephrine-chalone interaction.

1) If 1 hour has passed before crude epidermal extracts are added to incubating epidermal slices, no mitotic inhibition can be measured after a 4-hour Colcemid incubation. The extract must be added to the incubation early in the first hour for mitotic inhibition to occur, and the interpretation was that the extract protected the endogenous chalone-epinephrine complex.

2) After 5 hours in vitro, the mitotic depression caused by the epidermal extract or by epinephrine itself is reversed to control values.

3) Placing epidermis, previously incubated for 5 hours, into a fresh epidermal extract produced no mitotic inhibition.

4) Furthermore, if one placed epidermis, previously incubated for 5 hours, into fresh epinephrine for up to 1 hour, no inhibition occurred.

5) By placing epidermis into dilute epinephrine for as little as 5 minutes and then returning the epidermis to a 5-hour-old or freshly prepared epidermal extract, mitotic inhibition occurred.

In the above experiments, all extracts were >1 hour old when initially used, thus allowing tissue-bound epinephrine to have been completely oxidized or enzymatically hydrolyzed by monamine oxidase and catechol-O-methyl transferase. Marrs and Voorhees (*35–37*) have performed experiments similar to certain of those mentioned above, and their results confirmed the original work of Bullough and Laurence.

These facts indicate that epinephrine is probably of importance in G_2 mitotic inhibition. That beta adrenergic stimulation may be of importance in the G_1 phase of the cell cycle in ectodermal tissues is suggested by the fact that isoproterenol retards the flow of G_1 cells into the S phase of the cell cycle of eye lens epithelium (*38, 39*). To our knowledge, the effects of isoproterenol on epidermal G_1-to-S flow are unknown. Studies to

answer this question are under way in our laboratory.

EPINEPHRINE, CYCLIC AMP, AND EPIDERMAL MITOSIS

It has been known for some time that epinephrine acts by either increasing or decreasing cyclic AMP levels within cells (*40*). Since Vincent (*41*) had shown that norepinephrine, primarily an alpha adrenergic agent, did not inhibit epidermal mitosis and since Ryan and Heidrick (*42*) had shown that exogenously added cyclic AMP inhibited the growth of cells in culture, we predicted that epinephrine-induced epidermal mitotic inhibition should be associated with a beta adrenergic increase in cyclic AMP (*26*). In order to prove that a hormonally induced event—in this case epidermal mitotic inhibition—is mediated by cyclic AMP, it is necessary to fulfill four criteria as recently outlined by Robison, Butcher, and Sutherland (*43*). Our technology, statistical design, quality control, and results have been published (*3, 4, 21–32*). Therefore, our findings will be briefly summarized in this presentation.

The stimulation of adenyl cyclase in broken-cell preparations of epidermis by the beta adrenergic agonist, isoproterenol, is blocked by the specific beta adrenergic antagonist, propranolol (*28*). Mier and Urselmann (*44*), using full-thickness skin (an experimental approach with which we completely disagree due to the heterogeneity of skin—epidermis, fibroblasts, hair matrix cells, endothelial cells, etc.), found no catecholamine sensitivity of adenyl cyclase in broken-cell preparations. Whether this negative result was due to excessive beta receptor damage during homogenization or to tissue heterogeneity is unknown. The isoproterenol stimulation of cyclic AMP synthesis in intact epidermis within 5 minutes and inhibition of mitosis by isoproterenol within 5 hours are both blocked by propranolol (*4, 23, 32*). Brønstad, Elgjo, and Øye (*45*) have also shown that epinephrine stimulates epidermal cyclic AMP synthesis in intact epidermis but did not study its effects on mitosis or examine the question of alpha versus beta action in relation to epidermal cyclic AMP synthesis. We recently showed that norepinephrine plus propranolol (giving a primarily alpha stimulus) had no significant effect on epidermal cyclic AMP synthesis or mitosis (*32*).

We have shown that theophylline inhibits epidermal cyclic AMP phosphodiesterase and mitosis (*29*). Mier and Urselmann (*46*) recently showed that theophylline inhibits cyclic AMP phosphodiesterase prepared from whole skin. For the same reasons given above, the relevance of such a finding, from an enzyme prepared from whole skin, to our studies with the epidermal cyclic AMP phosphodiesterase is unclear. Mier and Urselmann did not study the effects of theophylline on mitosis in skin, but recently Marks and Rebien (*47*) in Heidelberg have confirmed our finding that theophylline at several concentrations inhibits epidermal mitosis. Furthermore, we have shown that theophylline and isoproterenol inhibit epidermal mitosis synergistically (*32*). Histamine at pharmacological concentrations (used in our studies as a tool rather than a simulator of a physiological situation) stimulates epidermal cyclic AMP hydrolysis and mitosis (*4*). Exogenously added dibutyryl cyclic AMP over a wide concentration range inhibits epidermal mitosis, thus mimicking an isoproterenol-induced endogenous increase in intraepidermal cyclic AMP (*30*). Butyrate and 5′-AMP do not inhibit epidermal mitosis. Marks and Rebien (*47*) have also confirmed the fact that dibutyryl cyclic AMP inhibits epidermal mitosis in a G_2 assay over a concentration range similar to ours.

Since these results appear to satisfy the majority of the four criteria of a cyclic AMP-mediated hormone action (*43*), we wish to suggest that epinephrine inhibits epidermal mitosis via a beta adrenergic-mediated increase in intraepidermal cyclic AMP. In addition, a very active cyclic AMP-dependent protein kinase exists in epidermis (*26*). Therefore, since an increase in cyclic AMP in other tissues produces its physiological response by activating such a kinase (*48*), we suspect that epidermal mitotic inhibition might also be the result of epidermal cyclic AMP-dependent protein kinase activation. Whether this kinase acts in G_2 at the level of transcription, translation, or post-translation to inhibit the production or physiological function of cell division proteins is unknown. Cyclic AMP-

dependent protein kinase may be able to phosphorylate microtubular subunits (*49*). Perhaps the kinase in epidermis can phosphorylate mitotic spindle microtubular subunits and prevent formation of a functional spindle, much as cyclic AMP kinase promotes the formation of a functionally inactive (dependent) glycogen synthetase (D) (*50*).

Although we have not directly measured endogenous epidermal cyclic AMP in the presence of histamine, the fact that histamine stimulates both cyclic AMP hydrolysis and mitosis suggests that decreased epidermal cyclic AMP results in increased mitotic activity (*4*). This seems especially plausible, since exogenously added dibutyryl cyclic AMP inhibits epidermal mitosis (*30*) as does epinephrine, which increases cyclic AMP in epidermis (*27*). Therefore, it appears that epidermal cyclic AMP levels and mitotic activity, as measured in our primarily G_2 cell cycle assay, are inversely proportional.

EPINEPHRINE-CHALONE SYSTEM INTERPRETED IN TERMS OF CYCLIC AMP

The early experiments of Bullough and Laurence (*33*) and our recent ones summarized above suggest that a naturally occurring molecule can slow the G_2 phase of the cell cycle. In our view, this G_2 control probably plays a minor role in the overall regulation of the cycle. Since the main problem is to delineate the nature of the proliferation-differentiation relationship, the G_1 phase of the cell cycle must contain the major decision-making control mechanism. Our G_2 experiments were done in an attempt to decide whether cyclic AMP might control both G_2 and G_1, a task which might not be difficult for a molecule with the diverse capabilities of cyclic AMP. If so, the conceptual difficulties which we mentioned earlier, of a G_1 control mechanism (the chalone concept) based on a G_2 assay, could be resolved. We have used psoriasis as a model of deranged G_1 control, since the most profound abnormality in the epidermal cell cycle of that disease is a marked shortening of G_1. This will be discussed later.

At this point we wish to examine the epinephrine-chalone concept in terms of our studies on the role of the cyclic AMP system in G_2. So-called chalone extracts are simply water-soluble epidermal extracts, the heterogeneity of which can be readily appreciated by examining the polyacrylamide gels reported by Marrs and Voorhees (*36, 37*). When one adds such an extract to an incubating ear slice, several possibilities exist:

1) One or several components of the extract activate epidermal adenyl cyclase directly via a chalone receptor in the epidermal cell surface. Such receptors might be a normal constituent of the epidermal cell surface (glycocalyx). The extract may need to be added early in the first hour of the experiment because a 5-hour incubation in a physiological solution is probably one of progressive metabolic decay.

2) The extract might enhance the effectiveness of tissue-bound epinephrine present at the onset of the experiment or inhibit cyclic AMP phosphodiesterase. This would be compatible with the fact that epidermal cyclic AMP is completely hydrolyzed 25 minutes after isoproterenol is added to epidermal slices (*4*).

3) The extract might enhance the activity of the cyclic AMP-dependent protein kinase.

4) The fact that a 5-hour-old epidermal slice must be briefly re-exposed to epinephrine before it will react, by mitotic depression, to an old or fresh epidermal extract suggests that the extract constituents are acting by enhancing the response of adenyl cyclase to epinephrine on the cell surface or by penetrating the plasma membrane and moving through to the cellular interior to inhibit cyclic AMP phosphodiesterase.

Obviously, many other possible explanations exist, including substrate depletions and intracellular specific substrate pool exhaustions. In any case, it seems quite possible that the work of Bullough and Laurence in 1964 (*33*) and of Marrs and Voorhees in 1971 (*35–37*) may be explained by an increase in intraepidermal cyclic AMP.

CYCLIC AMP SYSTEM IN NORMAL AND PSORIATIC EPIDERMAL PROLIFERATION AND DIFFERENTIATION

We have used psoriasis as a model of deranged

proliferation and differentiation because normal epidermal cells are extremely difficult to grow in vitro and, although organ cultures of adult skin are possible, the rate of G_1-to-S flow is unpredictable. The cell cycle is accelerated by a factor of approximately 12 in the epidermis of a lesion of psoriasis, all phases of the cycle being shortened. The most pronounced shortening is in the G_1 phase. Whether the short G_1 is the cause or the result of the abnormal keratinization of a psoriatic lesion is unknown. The proliferative compartment of a psoriatic lesion comprises only approximately one-third of the viable epidermal cells. The upper two-thirds of viable cells are involved in the abnormal keratinization of psoriasis and are therefore at least partially differentiated, a differentiation which in all probability occurs out of the G_1 phase of those cells cycling in the proliferative compartment (lower third) of the lesion.

Although it is possible to separate normal animal epidermis into its layers by various enzymatic hydrolyses (*51*), this is impossible in epidermis from psoriatic lesions. Therefore, the cyclic AMP measurements to be discussed below are from full-thickness psoriatic epidermis with the contamination of those dermal papillae which wander up and down between regions of psoriatic are from full-thickness psoriatic epidermis with cellular material in such a preparation is keratinocytes. Clearly then, psoriasis is not an ideal experimental "model" for studying the relevance of cyclic AMP to G_1 proliferative cells. We have used psoriasis for three reasons: 1) The relationship between G_1 and differentiation (keratinization) in the epidermis is intimate—both are deranged in psoriasis; 2) long ago, Bullough (*18*) suggested that the function of the chalone system might be to stimulate differentiation and thereby restrict proliferation [psoriasis is a superior candidate for a disease with an abnormal chalone mechanism; in fact, Shuster (*52*) recently suggested that psoriasis might be a "chalonosis"]; 3) we have a great interest in understanding the molecular pathology of the disordered proliferation and differentiation of psoriatic keratinization.

What is there to suggest that cyclic AMP may be involved in psoriatic keratinization? In the upper two-thirds of the psoriatic epidermis, glycogen is stored as seen by histochemistry (*13*). Biochemically, this is a threefold increase (*14*). The presence of cyclic AMP activates the enzymes associated with glycogen degradation and inactivates the enzyme associated with glycogen synthesis (*53*). The accumulation of glycogen in psoriatic epidermis would indicate an actual or functional decrease in cyclic AMP levels. We had shown that, in epidermis, isoproterenol, via a beta adrenergic increase in intraepidermal cyclic AMP, mediates isoproterenol-induced G_2 mitotic inhibition. Whether circulating catecholamines affect G_1-to-S flow is unknown, as is the possible neurobiological control of epidermal proliferation by the interaepidermal argentaffin-like system, the Merkel cells (*54–57*).

There is a diurnal variation in the labeling index of mouse epidermis, which has been shown to be the result of a changing flux of cells entering S from G_1 (*58*). Whether this is mediated by an increase in intraepidermal cyclic AMP is unknown. However, Ryan and Heidrick (*42*) and, later in a series of elegant studies, Pastan's group (*59–67*) showed that cellular proliferation could be specifically slowed by cyclic AMP. As will be discussed below, there is much to suggest that cyclic AMP slows cellular proliferation by enhancing differentiation. If one considers the epidermal hallmarks of psoriasis—incomplete differentiation, 12-fold accelerated cell division, and glycogen accumulation in the lesion (*3*)—in association with the above comments on the role of cyclic AMP in glycogen metabolism and cell division and differentiation, a strong circumstantial case could be made for a decrease in intraepidermal cyclic AMP levels in the lesions.

We therefore measured the levels of cyclic AMP in the epidermis of lesions of 25 patients with psoriasis and compared these levels to those of uninvolved epidermis from the same 25 patients. These values were also compared with cyclic AMP levels in the epidermis of 25 control volunteers. (The data were expressed in terms of pmol of cyclic AMP per mg Lowry protein, per μg DNA, and per mg wet weight because the involved and uninvolved areas are dissimilar.) The decrease in cyclic AMP in involved areas

compared to uninvolved areas is highly significant for all three data bases ($P < 0.0005$) (*3*). This decrease in cyclic AMP in the epidermis of lesions of psoriasis is in accord with and strongly supports our previous hypothesis of a defective cyclic AMP cascade as the molecular basis of the rapid epidermal kinetics of psoriasis. Furthermore, it seems quite likely that the decreased cyclic AMP levels will ultimately be shown to be metabolically significant. Experiments are in progress in our laboratory to examine this question of pathophysiological relevance.

Having reviewed the literature carefully, we are unable to offer an explanation other than a decrease in cyclic AMP to explain the constant association, in psoriasis and other proliferative dermatoses (including wound healing), of accelerated proliferation, incomplete differentiation, and glycogen storage. Later in this presentation we will suggest that cyclic AMP may instruct an epidermal cell to either divide or differentiate (a possible "switch mechanism"). Since the epidermis is morphologically and therefore biochemically stratified, one can offer an alternative to this "switch mechanism" hypothesis. Cyclic AMP levels could be decreased at all levels in the psoriatic epidermis. In the differentiated upper cells, a decrease could allow glycogen accumulation and incomplete keratinization, whereas a decrease in the lower third of the psoriatic epidermis could result in rapid proliferation by virtue of the absence of a cyclic AMP-induced G_1 braking mechanism. Said differently, the manifestations of decreased cyclic AMP could depend on the differentiated state of the epidermal layer in which the decrease occurs. We prefer the alternative "switch mechanism" notion which will be given later.

CYCLIC AMP SYSTEM IN PROLIFERATION AND DIFFERENTIATION OF OTHER TISSUES

Our work (*3, 4, 21–32*), the elegant studies of Pastan's group (*59–67*), and work by Sheppard (*68, 69*) and others (*38, 39, 70–88*) provide evidence that the cyclic AMP system may be important in growth control. To what extent this control is direct or indirect and the nature of its molecular bases have not been clarified as yet. It appears that low intracellular levels of cyclic AMP are associated with accelerated proliferation, and high levels are associated with decreased proliferation (*3, 64*). Also, recent data suggest that cyclic AMP may enhance differentiation in animals (*89–109*) and plants (*110–113*). Some investigators have documented decreased growth in association with enhanced differentiation, but others have not commented on whether proliferation was or was not decreased.

Also, in certain cells, cyclic AMP may stimulate proliferation (*114–124*). To our knowledge the endogenous levels of cyclic AMP have not been measured in such experiments and found to be high in such cells undergoing rapid proliferation. Rather, these experiments have been done by observing increased proliferation after certain cells (salivary gland, hematopoietic cells, and thymocytes) are exposed to added hormones and cyclic AMP. The physiological importance of either stimulation or inhibition of cell growth with cyclic AMP remains unknown. However, an examination of the literature and our own work suggests that cyclic AMP may in fact be important as one possible mediator of growth and proliferation.

The experimental designs used by the various workers mentioned above are very different in terms of hormone concentrations and timing of additions to the experimental material. Also, the differentiated and metabolic state of the cell in which the cyclic AMP increase or decrease occurs must be of signal importance to the outcome of such a change. It therefore seems unwise to attempt to decide at this time whether cyclic AMP stimulation or inhibition of cellular proliferation is the more important event. We wish to suggest that an increase or decrease in cyclic AMP levels may cause or permit a change to occur in the relationship between proliferation and differentiation in certain tissues. It should be apparent that, in nonproliferating tissues such as muscle or brain, a change in cyclic AMP levels cannot alter proliferation. To what extent, if any, cyclic AMP might play a role in the maintenance of the differentiated state of such nonproliferating tissues is unknown.

Our intuitive bias is that, in continuous cell renewal systems, which are highly differentiated

and very limited in function (such as epidermis), cyclic AMP may be of great importance in the maintenance of the balance between proliferation and differentiation. However, in tissues which renew primarily on demand, such as liver and kidney, and have a multiplicity of functions, cyclic AMP is less likely to influence proliferation directly. Therefore, our speculation is that cyclic AMP will ultimately be shown to regulate growth in some tissues but not in others. Also, whether cyclic AMP will be important in terms of growth control in a given tissue will, in our view, reside in the differentiated state of the cell rather than in the cell cycle per se.

CYCLIC AMP SYSTEM AS MAJOR REGULATORY MECHANISM IN EPIDERMAL PROLIFERATION AND DIFFERENTIATION: AN HYPOTHESIS

Our hypothesis, which portrays the cyclic AMP system as a mediator of epidermal growth, is based on the ideas and evidence reviewed above. Said differently, this hypothesis is an attempt to interrelate the original epinephrine-chalone mechanism (*33*), the presence of glycogen in regenerating epidermis (*2*), the evidence implicating cyclic AMP in the control of growth in various tissues and cells in culture, Crick's morphogenetic gradient hypothesis (*125*), and, lastly, our observations on the cyclic AMP system in normal mammalian epidermis (*4*) and in the rapidly proliferating epidermis of the human disease, psoriasis (*3*).

When epidermis is wounded, glycogen accumulates and later DNA synthesis and mitosis occur. This relationship is also seen in various proliferative epidermal disorders, chronic atopic dermatitis, and certain squamous cell carcinomas (*3*). Consequently, although we have limited cyclic AMP measurements to psoriasis because we consider it prototypic of disordered epidermal growth control, it is quite possible that cyclic AMP will be low in all situations in which there is active epidermal replication. We envision epidermal chalone as one or several constituents of the epidermal cell surface. This notion was first suggested by Seelich of Vienna and Øye of Oslo to Iversen (*126*). Alternatively, chalone could be a cyclic AMP phosphodiesterase inhibitor. In such a scheme, chalone in the epidermal cell surface would chronically maintain the "normal" epidermal adenyl cyclase activity by cell-cell interaction.

Since epidermis is rich in prostaglandin E_2 (*127*) and since prostaglandin E_2 stimulates epidermal adenyl cyclase (*128*), the control of normal epidermal adenyl cyclase activity may be orchestrated by cell-surface chalone, prostaglandin E_2, and systemically distributed hormones such as epinephrine or locally released neurotransmitters such as dopamine. The various hormones and chalone could regulate a single adenyl cyclase through different cell-surface receptors. This concept of multiple hormone receptors in a cell surface activating a single adenyl cyclase has been extensively studied by Rodbell's group in fat cell ghosts (*129*). In addition, the crucial role of the cell surface in growth control has been extensively studied and its importance reviewed by Burger (*130*).

In our model, any injury, no matter how minor, would perturb the spatial architecture or the molecular constituents of the epidermal cell surface, thus decreasing adenyl cyclase activity and, in turn, cyclic AMP levels. Such a decrease in cyclic AMP would then switch the synthetic processes characteristic of normal epidermal differentiation (keratinization) to a program of cell replication with its concomitant accumulation of glycogen. As soon as the newly formed cells entered G_1, certain of them would differentiate and produce normal levels of cyclic AMP and consequently normal glycogen levels. In this scheme, even very local or superficial damage could trigger proliferation in the basal cells—cells whose cycle reproduction genes are still available for transcription.

We envision cyclic AMP in our system as a morphogen, a concept suggested by Crick (*125*). Adapting Crick's notions to ours, the "source" of cyclic AMP would be the differentiated epidermal compartment and the "sink" would be the proliferative compartment. Cyclic AMP would diffuse to the "sink" (basal proliferative layer) through epidermal tight junctions or other specialized cell-surface structures and be destroyed in the basal layer. In its sojourn from source (differentiated compartment) to sink

(proliferative compartment), cyclic AMP would either stimulate or permit keratinization. In the normal basal layer, the cyclic AMP level would be sufficiently low to allow normal cell replication to occur. In Crick's hypothesis, in going from source to sink cyclic AMP would act as a morphogen over a concentration gradient of <1 mm. This also is in accord with damage-induced cyclic AMP deficiency. When epidermis is wounded, DNA synthesis (*131*) occurs over a distance of approximately 300 cells (1 mm) and mitosis (*132*) occurs over a distance of about 1 mm as well.

In summary, our hypothesis suggests that the cyclic AMP system plays a critical role in the regulation of epidermal proliferation and differentiation. In this scheme the role of cyclic AMP is to maintain the differentiated epidermal state. When damaged, the system "permits" cell division to occur, thus restoring normal epidermal architecture. The specificity of the system is ascribed to the cell surface, and we have used the word "chalone" to describe cell-surface glycoproteins. The word has been used because this is a chalone conference and because the word "chalone" brings to the mind of many investigators tissue specificity. However, in the future, as in the past, we will speak of the chalone concept, an elegant formulation proposed in 1962 by Bullough (*18*). But it serves no useful purpose to talk of chalone in molecular terms because in our opinion even the most rigorous attempts have not yielded a pure chalone or even several chalones from any organ in the body (*37, 133–137*). This may come as a surprise to some investigators, but in our review of the world's literature we can find no rigorous data to support the existence of one or several molecules which would satisfy the requirements of the concept proposed by Bullough in 1962 (*18*).

Our hypothesis encompasses the glycogen accumulation which is a hallmark of epidermal repair and regeneration. In addition, it may be that the subcellular activities of which cyclic AMP is capable are sufficiently catholic to explain both the G_1 and the G_2 control of proliferation postulated by Gelfant and Candelas (*138*). If so, an explanation might be at hand for the shortening of G_1, S, and G_2 which occurs in psoriasis. Lastly, this concept embraces an extensive body of literature which provides reasonably strong circumstantial evidence that cyclic AMP is involved in certain types of growth control.

To examine this hypothesis rigorously is obviously a colossal undertaking which we have just begun. It requires the isolation and purification of epidermal cell-surface macromolecules. Furthermore, the hypothesis requires that the various fractions obtained must be checked for their ability to stimulate cyclic AMP synthesis and to enhance epidermal differentiation and slow proliferation. This notion of examining the ability of cell-surface molecules to enhance epidermal differentiation is new to the chalone concept and requires the examination of the response of biochemical markers of epidermal differentiation to chalone candidates. Several such markers are available which should provide a valid epidermal differentiation assay.

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The Epidermal Chalone and Keratinizing Epithelia [1]

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SUMMARY—The main problems considered here are: 1) Do the stress hormones act directly on the epidermal chalone, and 2) how discriminative is the in vitro test for epidermal chalone using the "adrenaline wash" technique? The following account illustrates some of the discrepancies that were found when an in vivo confirmation was sought regarding the effects of epidermal chalone on visible mitosis.—Natl Cancer Inst Monogr 38: 61–68, 1973.

ONE OF the striking features encountered with epidermal chalone experiments is the differences that exist between in vitro and in vivo results. For example, in regard to the effect of epidermal chalone on the rate of entry into S phase, those who work with in vitro culture systems find an immediate response (*1–3*), whereas those who are injecting extracts into animals find a delay of up to 12 hours before a depression is recorded in the uptake of tritiated thymidine (*4, 5*). Similarly, to take just one example from the literature, it has been reported from in vivo studies on visible mitosis that the activity of an extract lasts for only one-half hour (*6*), while in vitro it has been shown to last for at least 9 hours at 38° C (*7*). It is obviously important to confirm in vitro results by an in vivo study in order to ascertain the fate of, and true reaction to, a substance in situ. The experimental study in the present work was a joint project with Dr. Erik Randers Hansen of the Royal Dental College, Copenhagen (*8, 9*). As a result of this work on tongue epithelium and ear epidermis, a challenge is made to the concept of epidermopoiesis being controlled by a single substance acting by means of a negative-feedback system.

Up to 1970, published work involving epithelia with high proliferative activity had been confined to epidermis which had been insulted by surgery (*10, 11*) or by the application of carcinogens (*5*). Therefore, a comprehensive experimental study has been begun on epithelia from different body sites, which normally have different sizes, mitotic rates, terminal differentiation, and function. This study is aimed at the formulation of some of the major problems concerning the regulation of mitotic activity in different epithelia with the purpose of linking cell replacement to some of the processes involved in differentiation and the consequent function of the tissue. The more relevant preliminary findings are included here with the view to considering the epidermal chalone in relation to the biology of the tissue and not as a separate entity. This study is a joint project with Dr. Enno Christophers of the Dermatological Department, the University of Munich.

In all in vivo experiments reported here, emphasis was placed on constant experimental conditions. For each experiment the mice were always the same sex and strain—always 4-month-old males—and the animal house conditions were

[1] Presented at the First Symposium of the International Chalone Conference, Brook Lodge, Augusta, Mich., June 5–7, 1972.

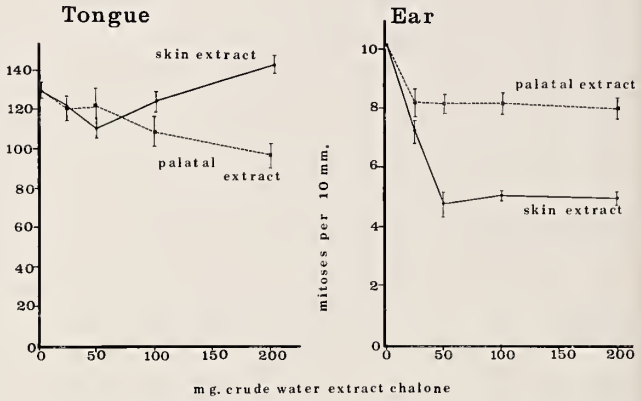
[2] I am pleased to acknowledge the grants awarded by the Wellcome Trust for technical assistance and expenses of the work in collaboration with Dr. Enno Christophers. I also wish to thank Mrs. Shirley Knibb, Fraulein B. Klahre, Mr. Paul Taylor, and Miss S. Heathfield for their assistance throughout this work.

constant so that feeding and cleaning were always done between 0930 and 1130 B.S.T. With this regimen, the mice were known to have a definite sleep period between 1100 and 1800. When animals are awake, the number of mitoses of the keratinizing epithelia is known to decrease (*12–15*), and so any environmental disturbance can give the same result as an injection of a mitotic inhibitor. All the experiments reported here pay special attention to this phenomenon. In addition, the epithelia being studied belonged to the same mice so that experimental conditions were always identical for all 4 tissues.

EPIDERMAL CHALONE AND STRESS HORMONES

It is remarkable that, when different amounts of epidermal chalone were injected into mice, the mitotic depression found in ear epidermis was never total and usually was in the order of 50–60%. This depression was reached when the equivalent of 50 mg of crude water extract of pig skin (2 mg of the active ethanol fraction) was injected per mouse. When the dose was increased by a factor of 6, no further depression was noted (text-fig. 1, *right*). Thus epidermal chalone is not the sole substance responsible for limiting mitotic activity. For instance, when physiological doses (up to 10 μg) of adrenaline were injected into each animal, there was a dose-dependent decrease in mitotic activity so that values of 75% and more were found for the mitotic depression. When physiological doses of hydrocortisone (up to 100 μg) were injected, only an insignificant depression occurred (*8, 9*).

In tongue epithelium of the same animals, the normal mitotic rate was approximately 10 times greater than that of the ear epidermis (text-fig. 1, *left*), yet the maximum mitotic depression with epidermal chalone was about 20%, and that maximum was reached when the equivalent of 50 mg of crude water extract of pig skin was injected (*8, 9*). This was surprising because it had been supposed that the normal high mitotic rate of tongue epithelium was due to a greater loss or to a lower rate of production of the epidermal chalone. The expected result, a greater response to a lower dose of added chalone, was not achieved. In addition, adrenaline gave a depression similar to that in ears (75%) but, in contrast to ears, hydrocortisone gave a significant depression of 21% (*8*).



TEXT-FIGURE 1.—Effect of increasing amounts of epidermal chalone extracted from pig palate (*broken line*) or pig skin (*continuous line*) on 4-hour Colcemid mitotic index. *Left:* tongue epithelium; *right:* ear epidermis.

All these experiments were performed on groups of 5 mice for each experimental procedure. The experiments were repeated 3 times and at no time were there any discrepancies, so pooling of results was justified.

It seems that epidermal chalone is not the primary factor maintaining the normal mitotic pattern of these two epithelia. The in vitro studies by Bullough and Laurence, on which the "adrenaline wash" assay is based, indicate that unless adrenaline is present in the tissues the epidermal chalone is ineffective. In further experiments, epidermal chalone was injected into adrenalectomized mice. Surprisingly, the same

mitotic depression was found in both tissues as when the epidermal chalone was injected into intact animals (*8*). Adrenaline and hydrocortisone gave greater mitotic depressions in the adrenalectomized animals, confirming the depletion of the naturally occurring stress hormones.

The results from these experiments do not support the conclusion, drawn from in vitro work, that adrenaline acts directly with epidermal chalone as a cofactor and some explanation compatible with all the results is necessary. Cell replication can be visualized and measured quantitatively; as a result, the chalone has been assigned the function of being primarily a mitotic inhibitor. This need not be so. It has already been suggested but not proved that the chalone might be primarily a promoter of cell maturity (*16*). A cell in the basal layer of the epidermis can either divide or proceed to maturity. In the presence of epidermal chalone, mitotic activity is depressed and the cell then becomes incorporated in the stratum spinosum and produces keratin precursors. Eventually, this cell is incorporated in the structural complexity of the keratinized stratum corneum, subsequently dying when it is sloughed.

In a tissue that is constantly challenged by being subjected to insult, there is needed a system which allows for the constant replacement of this tissue through, firstly, cell multiplication and, subsequently, differentiation. It has therefore been postulated (*17*) that a chalone antagonist exists, which allows mitosis to proceed, and it is on this chalone antagonist that the stress hormones act. The mitotic activity can be totally suppressed by adrenaline because the chalone-antagonist activity is removed and the endogenous chalone is allowed to work. Previous results (*17*) suggest that adrenaline either blocks or destroys the chalone antagonist while hydrocortisone prohibits its production. In normal circumstances this chalone antagonist is produced or mediated by the dermis and could be the reason that mitoses occur only in the basal layer.

Therefore, it seems that an epithelium with high mitotic activity could be produced hypothetically in several ways. A few that involve the epidermal chalone system may be: 1) by a lack of production or a direct loss of chalone through leakage from the epithelial cells or perhaps by a limitation of some factor influencing—e.g., the cyclic AMP cascade postulated by Voorhees' group (*18*); 2) by the direct biochemical inactivation of the chalone by the chalone antagonist; and 3) by the inhibition of terminal differentiation [as suggested by Christophers (*19*)], which may or may not be due to the chalone antagonist alone and which would result in persistence of either the immature or the undifferentiated state.

EPIDERMAL CHALONE FROM ORAL EPITHELIUM

In a review of the epidermal chalone (*20*), it was stated that, by using the in vitro discriminative test, epidermal chalone has been found in extracts of various epithelia including those of the mouth and the esophagus. It has been shown that this chalone, extracted from pig palate or gingiva, could be concentrated in the 71–80% ethanol fraction. This is the same fraction that is active when epidermal chalone is extracted from pig skin (*21, 22*).

When various doses of the two extracts were injected into C57Bl mice, together with Colcemid, the oral epithelial extracts depressed the mitotic rate of tongue epithelium more than did similar extracts of pig skin. Conversely, extracts of skin depressed the mitotic rate of ear epidermis more than did extracts of oral epithelium. This shows that the two extracts are not equivalent and that they are epithelia specific (*9*) (text-fig. 1).

At this point it is necessary to consider other aspects and experiments involving epithelia. The epidermis of the embryo may form stratified squamous keratinizing or nonkeratinizing epithelium. It may become mucus secreting. It may form part of the hair germ which may become outer root sheath, hair, or sebaceous gland. It may form the mammary, lachrymal, or salivary glands or the sweat glands. It may form part of the teeth and so on. Equally, tissues of nonepidermal origin may become stratified keratinizing epithelium—e.g., the esophagus and forestomach of the mouse (*22*). The importance of the underlying dermis in the formation of the subsequent type of epithelium has been the subject of much

experimentation (*23–25*). Apparently, the inclusion of the minutest part of the original underlying mesenchyme inhibits the ultimate transformation of an epithelium when it is grown on a foreign tissue of mesenchymal origin (*26*).

In its adult form, epidermis may become modulated by dermis to form the type of epidermis which has the structural architecture of the normal epidermis usually covering the host site. This has been shown by the elegant transplantation experiments of Billingham and Silvers (*27*) in which ear epidermis became structurally altered to form that of the sole of foot when it was transplanted to sole of foot dermis, and vice versa. However, when tongue and esophageal lining epithelia were transplanted to ear dermis, they retained their original form and structure. Ear epidermis and sole of foot epidermis are therefore labile tissues, whereas tongue and esophageal epithelia are more stable structures.

Thus the sebaceous glands and apocrine glands, although derived from epidermis embryologically, have become stable structures with their own chalone systems, whereas the tongue and esophagus apparently produce a chalone which can still act on the more labile ear epidermis but is still not as effective as the epidermal chalone derived from skin.

REGIONAL RESPONSE TO EPIDERMAL CHALONE

It may be argued that the moist environment of the tongue and the constant wear and tear exerted on the epithelium may be responsible for the differences in mitotic rate of the tongue and ear epithelia. For example, the tongue epithelium has a mitotic rate and duration very similar to those of wounded epidermis (*13*). In a comparative study being undertaken with Dr. Christophers, two regions of the skin with epithelia that vary considerably in their architecture (the footpad and ear epidermis) and two internal keratinizing epithelia (the epithelium of the undersurface of the tongue and that lining the esophagus) were chosen (fig. 1). As many parameters as possible are being measured on the normal tissues of 4-month-old male Swiss S mice, and a comprehensive experimental program which should affect some of these parameters is being undertaken.

Some of the more relevant results obtained so far are shown in table 1. The ear epidermis is a third to a quarter as thick as the other epithelia. It is already known that, when basal cells divide, the plane of division is perpendicular to the basement membrane so that both daughter cells are attached to the basement membrane (*28, 29*). By labeling with tritiated thymidine, it has been shown that, in the ear epidermis, both daughter cells are still on the basement membrane at the end of 5 days whereas in the other epithelia they have left the basement membrane and have traversed through the living stratum spinosum to reach the keratinized stratum corneum before that time. In ear epidermis it takes about 3 weeks before the first daughter cell reaches the cornified layer. Curves of percentage labeled mitoses obtained for all the tissues show that the S phase is the same (17–18 hr) for ear and footpad epidermis whereas it is much shorter for tongue epithelium and esophageal lining (9½–11½ hr). Similarly, the mitotic duration during the sleep period is of the same order for ear and footpad epidermis (3.3–3.8 hr) whereas it is much shorter for tongue and esophageal lining (1.3 hr). The labeling index and the mitotic index show that there is a considerably larger proliferative pool in footpad, tongue, and esophageal epithelia compared to ear. The size of the proliferative pool seems to be reflected in the speed of movement of cells through the epidermis, the wounded condition being an extension of the condition found in normal epithelia.

The effect of epidermal chalone on the visible mitoses is somewhat surprising (table 2). The results for tongue and ear epithelia are consistent with previous results, and an explanation has been suggested. Similarly, the esophagus apparently is even more stable and differentiated than tongue epithelium and so no longer reacts to epidermal chalone. However, Frankfurt (*5*) found some reaction of forestomach to extract of skin. The epidermis of sole of foot (with a high mitotic incidence and fast transit time but a mitotic timing similar to that of ear) reacted little to epidermal chalone extracted from pig skin. It is tempting to proceed with further speculation

TABLE 1.—*Comparative data on epithelia of ear, sole of foot, undersurface of tongue, and lining of esophagus*

Parameter	Mean ± SE*			
	Ear	Sole of foot	Tongue	Esophagus
Ratio, surface length/basement membrane length	1:1	1:1	1:1.2	1:1.1
Thickness of nonkeratinizing portion of epithelium (determined planimetrically) (μ)	13.9±0.9	46.8±2.5	54.2±2.7	49.7±1.9
Transit time of labeled basal cell to granulosa layer (days)	21	4–5	4–5	4–5
Labeling index/1,000 basal cells	50.9±2.6	270.3±22.2	68.0±4.2	122.0±8.4
Length of S phase (from % labeled mitosis curves) (hr.)	18.4	17.2	11.7	9.6
Mitotic index/1,000 basal cells	7.0±0.4	18.3±0.6	19.7±0.7	17.4±0.7
4-hour Colcemid mitotic index/1,000 basal cells	7.6±0.2	22.0±1.2	61.2±2.1	54.0±2.3
Duration of mitoses (hr.)	3.7	3.3	1.3	1.3

*Based on ten 4-month-old male Swiss S mice for each observation. The data have not been corrected by using the Abercrombie formula.

TABLE 2.—*Effect of epidermal chalone on mitosis in epithelia of ear, sole of foot, undersurface of tongue, and lining of esophagus*

	N*	4-hour Colcemid mitotic index/1,000 basal cells†			
		Ear	Sole of foot	Tongue	Esophagus
Control	15	7.5±0.2	20.6±1.3	62.4±1.8	56.8±2.3
120 mg 71–80% ethanol extract of pig skin (extracted from 300 mg of crude water extract)	10	2.4±0.2	12.7±0.6	47.5±4.1	58.8±4.1
P	—	<0.001	<0.001	<0.005	—
Percent depression	—	68	38	22	—

*Number of 4-month-old male Swiss S mice.
†Mean± SE.

but, first, far more observations and experiments and a far greater knowledge of the cellular action of the epidermal chalone are needed.

DISCUSSION

There is no doubt that the epidermal chalone is becoming really interesting as more and more evidence is accumulating. It seems that adrenaline and hydrocortisone do not act directly with epidermal chalone but appear to act on an intermediary substance. The "adrenaline wash" technique is not as discriminative as was expected, nor is the epidermal homeostasis regulated solely by a single substance acting by a single negative-feedback mechanism. That an epidermal chalone exists is incontrovertible. Its rightful place in the biology of epithelia is probably still obscure. The epidermal chalone had been assigned the function of being primarily a mitotic inhibitor because mitotic activity can be visualized and measured. This need not be so. It has already been suggested that it might primarily be a promoter of differentiation or maturity (*17*), thus allowing a differentiated tissue to develop. This could be its basic function in the embryonic state. Once this is postulated, then further substances or conditions are necessary to obtain tissue replacement and functional variations. Thus a careful balancing of these substances is necessary to maintain the steady state experienced in normal epithelia.

It seems reasonable that a basic pattern should be common to all tissues. Thus the site-dependent

variations experienced in epidermal differentiation apparently are secondarily imposed on the original chalone pattern. From other tissues, an antichalone has already been found for granulocytes (*30, 31*) and erythrocytes (*32*). Fibroblasts need a serum factor (*33*) before they will grow in culture. Similarly, epidermis needs a factor produced by fibroblasts (*34*) for effective long-term in vitro culturing. Perhaps the role of inflammation, which has been the subject of past conferences at Brook Lodge (*35, 36*), will subsequently be linked with the epidermal chalone in the healing of skin wounds.

A study of epithelial tissue kinetics is of considerable interest here. The differences in speed of movement of cells through the immature living epidermis to the mature and functioning cells of the stratum corneum in various epithelia may be more important than was at first envisaged. The mere act of slowing down this motility of cells [a feature already attributed to cyclic AMP with fibroblasts (*37*)] and their retention in the basal layer are only some of the many parameters not explored so far in relation to epidermal chalone.

There is already enough speculation about chalones to keep experienced biologists, pathologists, and biochemists busy for a very long time, but it is important to constantly challenge the results and interpretations to be sure that what is actually measured is not a by-product of the true primary reaction. The difficulty always is in identifying the proverbial "red herring."

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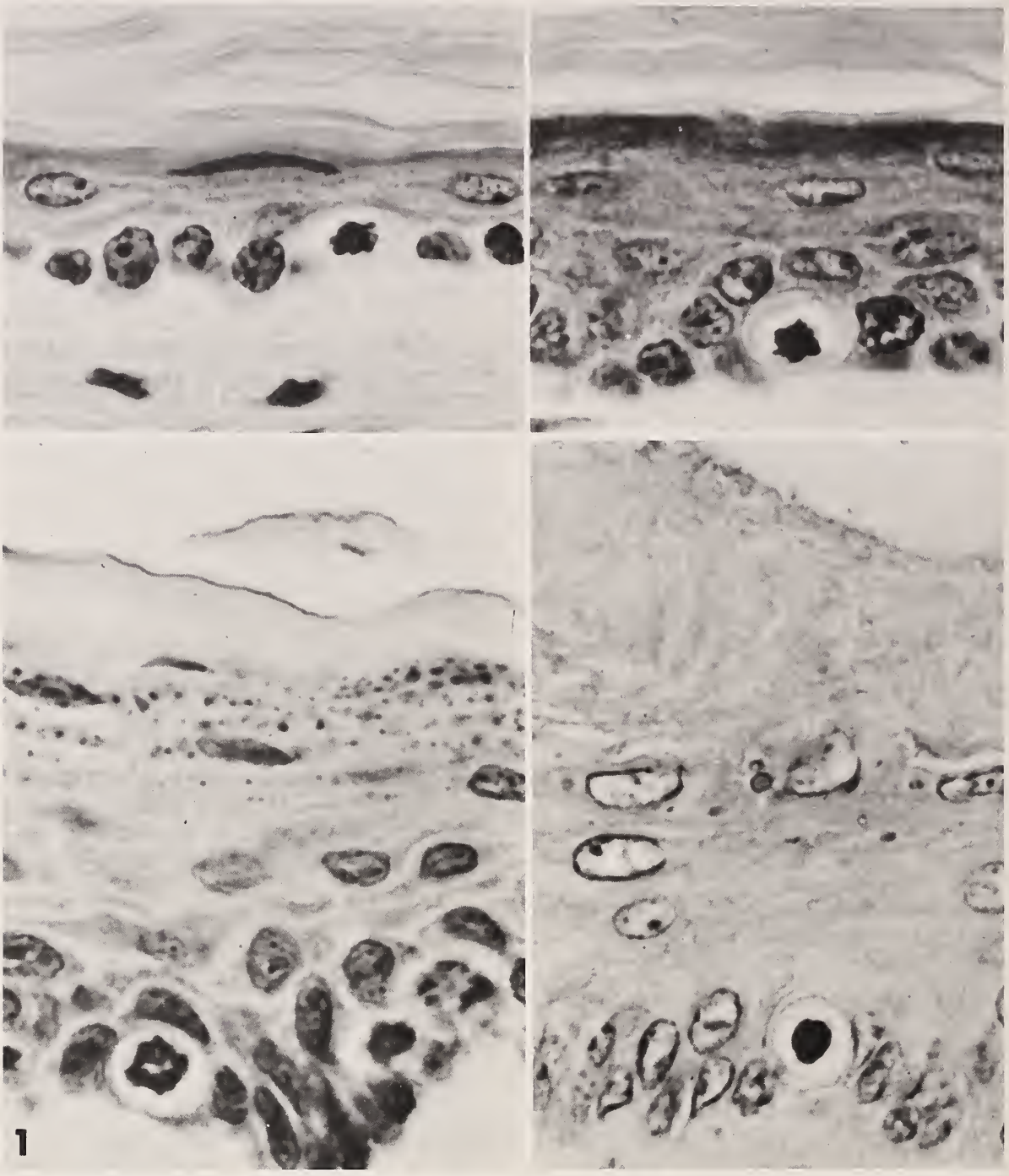

FIGURE 1.—*Upper left:* ear epidermis; *upper right:* epidermis of sole of foot; *lower left:* epithelium of undersurface of tongue; and *lower right:* epithelium lining of esophagus. All sections are from same mouse, and all photographs are at same magnification (× 1,300) to show comparative sizes and structures of epithelia. Colcemid-arrested mitoses appear in basal layers of all epithelia. Ehrlich's hematoxylin and eosin.

Commentary on "The Epidermal Chalone and Keratinizing Epithelia" by E. B. Laurence [1]

Henry Hennings, *Experimental Pathology Branch, National Cancer Institute,*[2] *Bethesda, Maryland 20014*

A MAJOR PROBLEM inherent to all chalone experiments is that one must work with more or less crude tissue extracts. It is difficult to perform meaningful experiments with such heterogeneous mixtures of biological material. However, the experiments described by Dr. Laurence regarding the site specificity of related epithelial chalones in the ear, tongue, sole of foot, and esophagus can yield useful results even with relatively crude extracts.

With regard to the site-dependent differences in response to epidermal chalone, I would like to mention some results obtained by Dr. Ole Nome of the Institute for General and Experimental Pathology, Rikshopitalet, in Oslo. In Dr. Nome's experiments, mice were given Colcemid and a tissue extract intraperitoneally 3 hours before they were killed. With aqueous extracts from skin, an appropriate dose depressed the mitotic rate by 70% in the interfollicular epidermis but by only 27% in the forestomach. However, with forestomach extracts, the depression of mitotic rate in the epidermis was decreased to 52% whereas the depression in the forestomach was increased to 37%. The mitotic rate in the small intestine was not significantly decreased by injection of either extract. These results support Dr Laurence's suggestion of related epithelial chalones.

Dr. Laurence's finding that the epidermal chalone depresses the mitotic rate to an equal extent in normal and in adrenalectomized mice is significant. As an alternative to Dr. Laurence's suggestion that adrenaline and hydrocortisone may affect chalone action indirectly by inhibiting a chalone antagonist, this result could be interpreted to indicate that the stress hormones may be unnecessary for chalone action in vivo. As Dr. Marks will tell us later in this conference, the epidermal factor which acts in the G_1 phase of the cell cycle does not require adrenaline for activity. A literature survey indicates that chalones acting in G_2 to depress the mitotic rate generally need adrenaline for activity, while chalones acting in G_1 to inhibit the entrance of cells into the S phase generally act in the absence of adrenaline. In vivo experiments with hydrocortisone and epidermal chalone revealed no synergism in the inhibition of DNA synthesis by these two factors (*1*). The possible role of the stress hormones in the action of epidermal chalone remains to be clarified by further experimentation.

I have two questions for Dr. Laurence. I think that most of us would agree that the most logical place for a physiological growth regulator to act would be in G_1, controlling the entrance of cells into the phase of DNA replication. Your interesting experiments on the response of different epithelia to different epithelial chalones were performed using a G_2 assay, estimating a depression in the mitotic rate. Do you plan to repeat these experiments using a G_1 assay?

[1] Presented at the First Symposium of the International Chalone Conference, Brook Lodge, Augusta, Mich., June 5–7, 1972.

[2] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

My second question is with regard to the chalone antagonist (or antichalone) which you have suggested may be made in the dermis and the synthesis of which is controlled by the stress hormones. What is known about the effects of the stress hormones on the dermis?

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Epidermal Chalone: Cell Cycle Specificity of Two Epidermal Growth Inhibitors [1]

Kjell Elgjo, *Institute of General and Experimental Pathology, Rikshopitalet, Oslo 1, Norway*

SUMMARY—Injection of lyophilized crude water extracts of whole epidermis will inhibit epidermal cells in the G_2 phase and cells in the late part of the G_1 phase (the presynthetic phase). This double effect seems to be mediated by two different inhibitors that can be separated from each other in different ways. Thus, after topical treatment of the skin with actinomycin D, the G_2 inhibitor will disappear from the treated area within a few hours, while the G_1 inhibitor is present even at 24 hours after the treatment. When the epidermal cells are separated by means of trypsin, the G_2 inhibitor can be extracted from the basal cells and the G_1 inhibitor, from the keratinizing cells. On the basis of the experiments reviewed in this article, it is proposed that 1) a factor with a relatively short turnover time and acting on cells in G_2 is present in or produced by the epidermal basal cells, and 2) a component with a longer turnover time is produced by the keratinizing cells and regulates the rate of entrance of cells from G_1 to S.—Natl Cancer Inst Monogr 38: 71–76, 1973.

THE FIRST REPORTS on the epidermal chalone indicated that this mitosis-inhibiting factor was 1) a thermolabile, water-soluble, nondialyzable protein (*1, 2*), 2) tissue-specific but species-nonspecific (*3*), and 3) produced locally in the epidermis where it acted on the proliferating basal cells (*4*). A number of questions remained, and still remain, unanswered. Among the more salient unsolved problems are the following: What is the exact chemical nature of the mitosis-inhibiting factor? Which cells synthesize the inhibitor, and where and how is it broken down? On which phases of the cell cycle does the epidermal chalone act? Is there only a single mitosis-inhibiting factor, or does the epidermal chalone consist of several different components?

This paper reviews some findings that could indicate the answers to some of the questions. But, because the epidermal chalone has not yet been fully purified, all results must be regarded as preliminary. Contaminating substances present in the extracts possibly could modify the results.

[1] Presented at the First Symposium of the International Chalone Conference, Brook Lodge, Augusta, Mich., June 5–7, 1972.

MATERIALS AND METHODS

Animals.—Hairless mice, *hr/hr,* of both sexes and about 3 months old were used both for preparing and for testing the various extracts.

Preparation of tissue and cell extracts.—Whole mouse skin was homogenized in a mill cooled by liquid nitrogen, and water extracts were prepared as described previously (*5*). Separated epidermal cells were homogenized in a porcelain mortar cooled with liquid nitrogen, and water extracts were prepared as described in previous papers (*6, 7*). The temperature was kept at 0–4° C or below during all procedures. Unless otherwise stated, all extracts were lyophilized and stored at −20° C until they were used.

Separation of epidermal cells.—The basal cells were separated from the differentiating epidermal cells according to the method described by Lærum (*8*), but instead of the separated cells be-

ing put into a buffer they were frozen in liquid nitrogen immediately after the separation procedure (7).

Estimation of the mitotic rate.—The epidermal mitotic rate was tested in vivo by means of the Colcemid technique. After intraperitoneal (ip) injection of 0.15 mg of Colcemid, epidermal mitoses are arrested for the following 4-hour period. The number of accumulated mitoses gives an estimate of the number of cells beginning their division per unit time in the examined cell population. In all experiments, mitoses arrested by Colcemid in 4 hours were counted per 8 mm of interfollicular epidermis.

Estimation of epidermal DNA synthesis.—Epidermal DNA synthesis was estimated either by measurement of the incorporation of tritiated thymidine into epidermal DNA or by autoradiography. In both cases the mice were given ip injections of 30 μCi of tritiated thymidine (specific activity, 6.7 Ci/mmole, New England Nuclear Corporation) and killed by neck fracture 30 minutes later. With the first method, the specific activity of epidermal DNA was calculated as described previously (*5*) and expressed as cpm/μg DNA. Sections for autoradiography were dipped in Kodak NTB2 emulsion and exposed for 4 weeks at 0–4° C. Labeled cells were counted in 8 mm of interfollicular epidermis [for details, *see* (7)].

RESULTS AND DISCUSSION

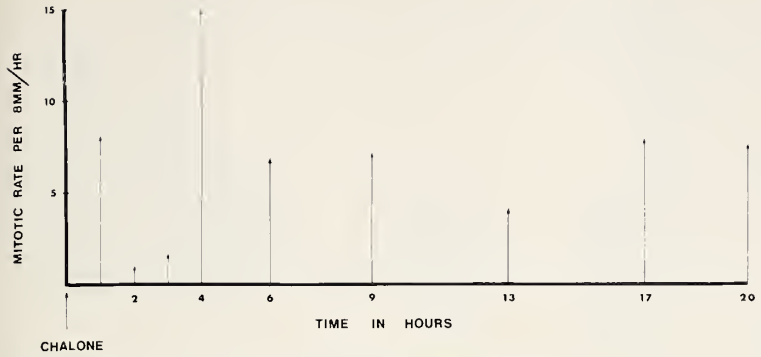
Phases of the Cell Cycle Inhibited by the Epidermal Chalone

All early work on the epidermal chalone was made in assay systems that were based upon the Colcemid technique. As mentioned above, Colcemid will arrest epidermal mitoses for 4 hours after injection of this stathmokinetic agent. The duration of the G_2 period in the hairless mouse epidermis is, on the average, 4 hours. This means that the Colcemid technique, as used in these early experiments, will register only the effects on the rate of passage of cells from G_2 into mitosis. Therefore, to examine this in more detail, we injected 5 mg of lyophilized crude skin extract ip and estimated the mitotic rate from hour to hour in different groups of mice. These groups had received Colcemid at different times after the injection of the skin extract. The detailed procedures and the findings have been published previously (*9*).

Text-figure 1 summarizes the effect, on the epidermal mitotic rate, of a single injection of 5 mg of crude skin powder. The mitotic rate was normal during the 1st hour but decreased to almost zero during the 2d and the 3d hours. By 4 hours, the mitotic rate was higher than normal. After a period of about 9 hours with a normal mitotic rate, there was a second, transient, decrease. Our interpretation of these results was that the epidermal chalone had no effect on cells in late G_2. However, the cells in the middle part of G_2 (about 2–3 hr before they would enter visible mitosis) were delayed or arrested. Apparently this delay resulted in a partial synchronization so that more cells than normal started their mitosis about 4 hours after the injection of skin powder. The high rate at 4 hours did not quite compensate for the low rates at 2 and 3 hours. Some of the G_2 cells were therefore apparently lost in the sense that they were either prevented from continuing their division or started it later on and over such a long period of time that they did not increase the estimated mitotic rates.

The second decrease in the epidermal mitotic rate at 13 hours could indicate that the injected extract influenced processes that take place several hours before the actual cell division. This finding was interesting because it did not appear reasonable that the epidermal chalone should exert its main effect on cells in the G_2 period. Theoretically, it should act on some part of the G_1 period when cells decide whether to start the preparations for a new division or not [named the "dichophase" by Bullough (*10*)]. To test this, epidermal DNA synthesis was estimated at intervals after a single and after repeated ip injections of a skin extract we knew was active when tested with the Colcemid technique.

Text-figure 2 shows that the incorporation of tritiated thymidine was normal for about 8 hours after a single injection of skin extract. It then decreased for a period of 3–4 hours before it returned to normal values again. After 4 injections of skin extract there was again a delay of about



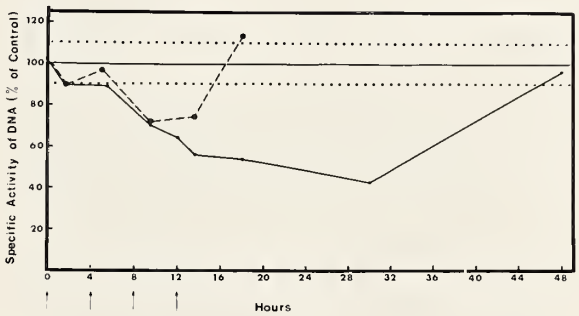
TEXT-FIGURE 1.—Groups of mice received 5 mg of skin extract ip at the time indicated by "chalone." The epidermal mitotic rate per hour per 8 mm of interfollicular epidermis was estimated by injecting Colcemid at different intervals [for details, *see* (*9*)]. The normal mitotic rate is 8.5 ± 0.9 per hour per 8 mm interfollicular epidermis.

8 hours before the epidermal DNA synthesis decreased (*5*).

The interpretation of this series of experiments was that the epidermal chalone had a strong inhibitory effect on processes going on in cells that were in the late G_1 period (the presynthetic phase) at the time when the extract was injected. It had no significant effect on cells actually in the S phase. When the results obtained with the Colcemid technique are combined with those found by measuring the incorporation of ^{3}H-labeled thymidine into epidermal DNA, the two series of experiments suggest that the skin extracts contain factors that inhibit cells in both G_2 and the presynthetic phase (late G_1).

Different Inhibitory Components in Mouse Skin Extracts

After a sudden cell loss the epidermal cell population reacts by an increased rate of cell proliferation after a delay of only a few hours. Such sudden cell loss occurs after wounding of the skin (*11*) and after application of hyperplasia-inducing agents (*12*). This could suggest that the mitosis-regulating factor or factors are continuously produced and broken down at a fairly rapid rate. To get an estimate of the rate of turnover of the epidermal chalone, actinomycin D in acetone was applied topically to inhibit epidermal RNA synthesis. Groups of treated animals were killed at intervals after a single application of 100 μg of actinomycin D, and the treated area was homogenized and extracted as described above. The extracts were tested for their effect on cells in G_2 by means of the Colcemid technique



TEXT-FIGURE 2.—Epidermal DNA synthesis after ip injection of a single (_____) or repeated (_____) doses of 5 mg of skin extract, expressed as percentage of the water-injected controls. Usual experimental range of $\pm 10\%$ is indicated for the control group (. . . .). *Arrows* indicate the injection times in the mice which received 4 injections. [Reproduced from (*5*).]

and for their effect on cells in late G_1 by estimating the incorporation of ^{3}H-labeled thymidine into epidermal DNA.

Text-figure 3 shows that the effect of the extracts on the cells in G_2 disappeared after 13 hours, while the inhibitory effect on cells in the presynthetic phase was present even at 24 hours after the application of actinomycin D. At that time, epidermal RNA synthesis was depressed

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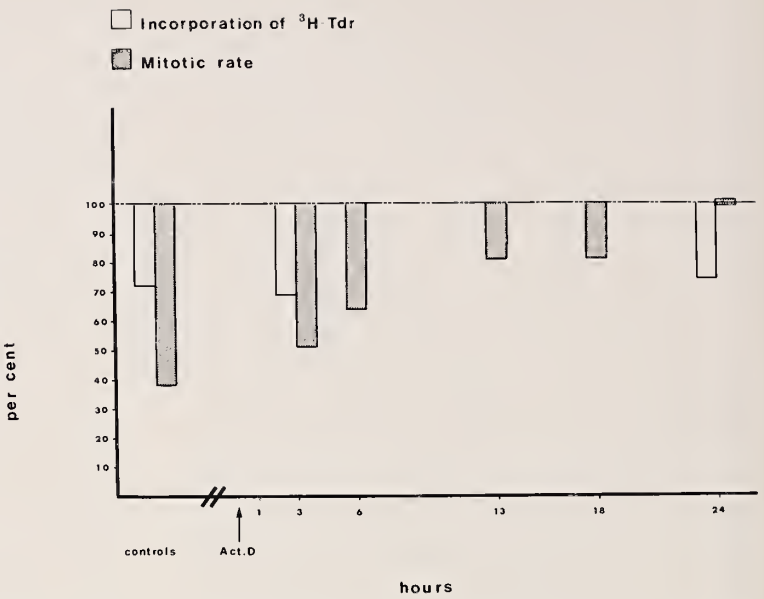


TEXT-FIGURE 3.—Mice were treated topically with 100 μg of actinomycin D, and extracts were made from the treated area of animals killed at intervals after treatment. Extracts were tested for their effect on cells in G_2 by means of the Colcemid technique and for their effect on cells in late G_1 by estimating the specific activity of epidermal DNA after injection of 30 μCi of ^{3}H-labeled thymidine (^{3}H-Tdr). In both cases, the effect is expressed as percentage of saline-injected controls. Statistically, the inhibition of the mitotic rate induced by extracts made from actinomyein D-treated skin is not significant at 13 hours and later, while the inhibition of epidermal DNA synthesis is significant in all groups. [For details, *see* (*13*).]

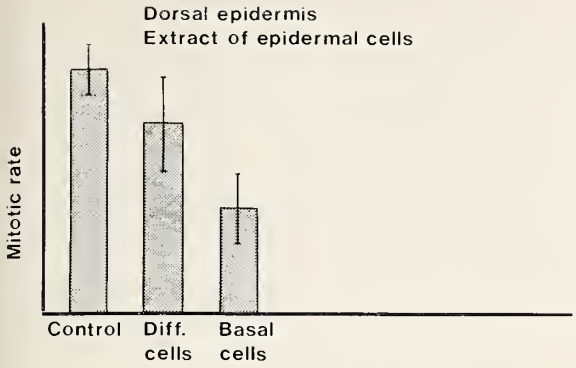
about 40% (*13*). These findings suggested that some part of the active component had, as expected, a fairly rapid rate of turnover. It also revealed, however, that there were probably at least two inhibitors present in the skin extracts—one that acts mainly on cells in G_2, and another that inhibits cells in the presynthetic phase. After treatment with actinomycin D, the two components were dissociated; the component acting on cells in G_2 disappeared within a few hours, while the substance acting on cells in the presynthetic phase was more stable or was coded for by a more stable RNA.

Theoretically, the epidermal chalone should be produced by the mature, differentiating cells. To test this, the basal cells were separated from the differentiating cells, as described above. The extracts obtained from the separated cell batches were tested for their effect on cells in G_2 by means of the Colcemid technique and for their effect on cells in the presynthetic phase by means of tritiated thymidine. In the latter series of experiments, both the incorporation of ^{3}H-labeled thymidine into epidermal DNA and the number of labeled cells were registered (*7*).

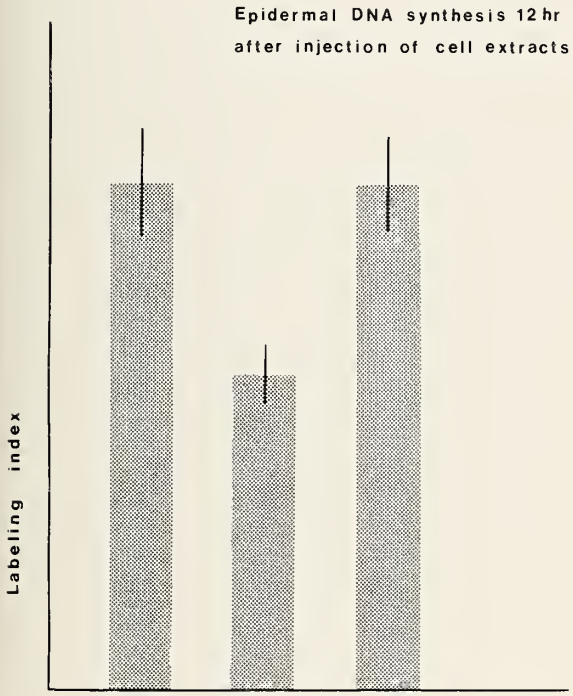
Text-figure 4 shows that the basal cell extracts strongly inhibited cells in G_2, while the differentiating cell extracts had only a varying and insignificant effect on that phase of the cell cycle. When tested for their effect on cells in the presynthetic phase, the results were reversed (text-fig. 5). The differentiating cell extracts induced a very significant depression of epidermal DNA synthesis after the usual delay of about 8 hours. The basal cell extracts had no significant inhibitory effect on this part of the cell cycle.

The interpretation of these findings was that the component present in the differentiating cell extracts seems to represent the epidermal chalone proper. It is apparently produced by the keratinizing cells and it acts where a chalone should act, on cells in the presynthetic phase. The finding of an inhibitor present in the basal cell extracts that acts on cells in G_2 was more puzzling. It is known, however, that the G_2 period can represent a second resting phase (*14*), and that the duration of this phase can vary considerably. Thus the G_2 inhibitor could well be related to the observations by Gelfant (*15*) that many cell populations have a discrete G_2 population that can react to stimuli in a more or less autonomous way. Recent work has also shown that, after application of a carcinogen to the mouse skin, there is a transient dissociation between the estimated contents of the G_2 inhibitor and the G_1 inhibitor in the treated epidermis (*16*).

When combined with the actinomycin D experiments mentioned above, the results could



TEXT-FIGURE 4.—Effect on the epidermal mitotic rate, as estimated by means of the Colcemid technique, of extracts made from separated epidermal basal and differentiating cells. [For details, *see* (*6*).]



TEXT-FIGURE 5.—Effect on epidermal DNA synthesis of extracts made from separated epidermal basal and differentiating cells. The mice received the various cell extracts (ip injection) 12 hours before death. This figure shows only the registered effect on the labeling indices. [For details, *see* (*7*).]

indicate that 1) a factor with a relatively short turnover time and acting on cells in G_2 is present in or produced by the basal cells and 2) a component with a longer turnover time is produced by the differentiating cells and regulates the rate of entrance of cells from G_1 into the S phase.

Recent reports have corroborated this hypothesis. Marks (*17*) has shown that crude pig skin extracts contain two factors that can be separated from each other by various fractionation procedures. One of the two components has a fairly high molecular weight and acts mainly on cells in the presynthetic phase, while the second factor has a lower molecular weight and acts mainly on cells in G_2. Similarly, Bichel (*18*) has found that Ehrlich ascites tumor cells produce two different inhibitors that act on cells in G_2 and on cells in late G_1, respectively. It is, however, much too early to tell if these findings represent a general physiological principle.

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Commentary on "Epidermal Chalone: Cell Cycle Specificity of Two Epidermal Growth Inhibitors" by K. Elgjo [1]

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I AM PRESENT at this meeting merely as one who has been concerned with living cells for a long time and to whom chalones are therefore of great interest. I must emphasize that I have never worked in this field, and so my comments are merely those of an unsophisticated onlooker.

I found Dr. Elgjo's paper exciting, and particularly his experiments on the inhibitory effect, on DNA synthesis, of extracts of the differentiated layers of the epidermis. I have a special reason for mentioning this finding, because I think it has a bearing on a rather puzzling observation that I made some years ago in experiments on the effect of excess vitamin A on the scaly metatarsal skin of 13-day chick embryos (*1*). I grew the skin as organ cultures. In control medium the epidermis keratinized and mitosis was restricted to the basal layer in the usual way; but, in the presence of a small dose of vitamin A (about 1 μg/ml of medium), keratinization was suppressed, mucous metaplasia was induced, and mitosis appeared at all levels, even in the superficial mucus-secreting cells. In the light of Dr. Elgjo's results, this suggests that, in the absence of keratinization, chalones either are not synthesized or are destroyed.

One of the questions to which Dr. Elgjo would like to find an answer is: Where and how is the inhibitor broken down? In one of his publications (*2*) he wrote: "The breakdown of chalone could be brought about by a special enzyme system, or it might be 'used up' by being bound to specific sites in the progenitor cells." I should like to consider briefly the first suggestion: That it is destroyed by a special enzyme system. It seems to me that the enzymes most likely to be concerned in the breakdown of the chalone would be the lysosomal hydrolases (*3*). Lysosomes are cytoplasmic organelles containing many hydrolases in a bound form. These enzymes can act both within and outside the cell. When material is taken into the cytoplasm by endocytosis, lysosomes fuse with the membrane-bound endocytotic vacuoles and tip their enzymes into the engulfed material which is then digested. Components of the cell's own cytoplasm may be destroyed in a similar way. For example, there is evidence that the secretory activity of the pituitary is regulated by a lysosomal mechanism [*see* (*4*)] in response to appropriate hormonal stimuli; secretory granules fuse with lysosomes and are then digested. An overproduction of the secretory product is thus prevented.

Lysosomal hydrolases are released into the cell's environment in two ways: by necrosis and, much more efficiently, by secretion. Under various abnormal conditions, the synthesis and secretion of lysosomal enzymes are greatly increased. My colleagues and I have studied this phenomenon in chick limb-bone rudiments in organ culture. Two agents that, in sublethal concentrations, increased the secretion of lysosomal enzymes in these explants were vitamin A (*5*) and an appropriate complement-sufficient antiserum (*6*). As might be expected, in view of the

[1] Presented at the First Symposium of the International Chalone Conference, Brook Lodge, Augusta, Mich., June 5–7, 1972.

increased enzymic activity, both agents caused degradation of the cartilage matrix. What we did not expect, however, was that both agents also induced the hypertrophic chondrocytes, which normally do not divide, to multiply quite actively by mitosis (*7,8*). There are, of course, several possible explanations of this surprising result but, after reading Dr. Elgjo's paper, I asked myself whether perhaps the overproduction of lysosomal enzymes had destroyed the chalones of the hypertrophic cartilage.

The hypothesis that I have proposed, that the lysosomal system is responsible for the destruction of chalones, is a very obvious one and must have been considered by some of those working on these mitotic inhibitors. On the other hand, none of the contributors to our large book on lysosomes (*3*) referred to research on these lines. It will be very interesting to hear what Dr. Elgjo and members of this conference have to say about this matter and whether they think that the possibility of a functional relationship between chalones and lysosomes deserves investigation as an alternative to the concept of antichalones.

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A Tissue-Specific Factor Inhibiting DNA Synthesis in Mouse Epidermis [1]

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SUMMARY—In this communication I shall report on experiments demonstrating that skin, the classical tissue of chalone research, probably contains an epidermis-specific G_1 inhibitor which is not identical with the adrenaline-dependent G_2 inhibitor, the so-called epidermal chalone. Also I shall outline attempts at the purification and biochemical characterization of this factor. The evidence suggests that the G_1 inhibitor is a glycoprotein with an apparent molecular weight of 100,000–300,000 Daltons and consisting of a large protein "coat," which can be removed by proteolytic digestion, and a small active "core," probably a glycopeptide, with a molecular weight of 10,000–20,000 Daltons. During lyophilization the factor seems to form large aggregates.—Natl Cancer Inst Monogr 38: 79–90, 1973.

THE HYPOTHESIS of chalone action developed by Bullough (*1*) on the background of the antitemplate theory of Weiss and Kavanau (*2*) includes, as an essential postulate, that chalones act primarily by inducing and maintaining cell functionalization. As a consequence, Bullough assumed, the cell is prevented from entering the phase of DNA replication. This block is thought to occur in the late G_1 phase of the cell cycle—called "dichophase" by Bullough (*1*)—when the cell has to decide between entering a new mitotic cycle or taking the path toward functionalization. Since, in normal tissues, functionalization from the G_1 phase is the common mechanism whereas functionalization from the G_2 phase seems to be a rather rare event, this hypothesis is highly plausible and consistent with experimental data.

Nevertheless, in most cases the effect of tissue-specific mitotic inhibition has been measured by using the Colcemid technique. By this method, however, only a block in the G_2 phase can be observed. Factors have thus been detected in several tissues—e.g., epidermis (*1, 3–5*), melanocytes (*6*), lymphocytes (*7*), lung (*8*), and kidney (9) as well as in some tumors (*6, 7, 10–12*)—which, together with adrenaline, inhibit the cell from going through mitosis by blocking the G_2 to M transition. An effect of these factors upon DNA synthesis (by inhibiting the G_1 to S transition) has been postulated by Bullough (*1*) but, until now, experimental proof of this hypothesis has been lacking. Convention has even succeeded, in a part of the literature, in developing a concept of "chalones" by which they are considered to be tissue-specific G_2 inhibitors which need adrenaline to be fully active. This is, of course, in sharp contrast to Bullough's original theory.

On the other hand, tissue-specific factors have been described which prevent cells from entering the S phase (G_1 inhibitors). Such inhibitors have been found in granulocytes (*13, 14*), erythrocytes (*13*), lymphocytes (*15–17*), and liver (*18*) and also

[1] Presented at the First Symposium of the International Chalone Conference, Brook Lodge, Augusta, Mich., June 5–7, 1972.

[2] This work was supported by the Deutsche Forschungsgemeinschaft. I gratefully acknowledge the skillful technical assistance of Miss U. Öhlers, Mrs. U. Schmid, and Miss I. Raab.

have been called "chalones," although they do not seem to depend on adrenaline.

Some confusion has thus arisen, not only regarding the definition of a "chalone" but also in respect to its biochemical action and its biological role in the maintenance of mitotic and functional homeostasis. The only way to escape these difficulties is the approach of extensive purification and biochemical characterization of chalone-like substances, which was started by the pioneering work of Hondius Boldingh and Laurence (*5*) on the epidermal G_2 inhibitor.

METHODS

Pulse-labeling with tritiated thymidine is still the most convenient and most widely used method for measuring DNA synthesis in a tissue. In order to estimate the DNA synthesis in mouse epidermis, we adopted the method of Hennings et al. (*19*).

However, the inhibition of thymidine incorporation into tissue DNA can be due to at least two different mechanisms:

1) The DNA synthesis itself is inhibited.

2) The cell or nuclear membranes—i.e., the permeability for thymidine—is altered in such a way that the labeling of the internal thymidine pool is delayed.

If the latter is a true explanation for the observations described in this communication, the alteration of membrane permeability would be a highly specific effect with a pronounced lag phase. Although such a mechanism cannot be excluded yet, we as well as other investigators in this field tend to favor the first possibility, believing the decrease of thymidine incorporation to be a true reflection of diminished epidermal DNA synthesis.

It has been shown by autoradiographic techniques that after relatively short pulses of tritiated thymidine (*20*)—or labeled uridine (*21*)—the incorporation of label is localized exclusively in the cells of the interfollicular epidermis of the mouse. The cells of the dermal layer are virtually unlabeled and consequently do not contribute appreciably to the radioactivity found in the DNA isolated from mouse skin as described below.

To be sure that DNA synthesis in the hair follicles does not falsify the results, only mice 7–9 weeks old were used in the experiments. At this time the hair follicles are already degenerated [telogen phase (*22*)]. The back skin of the animals was shaved at least 3 days prior to the experiment and only those mice were used which did not show a regrowth of hair. The radioactivity found under these conditions is attributable almost entirely to labeling of the interfollicular epidermis.

We performed our experiments as follows. Female mice (strain NMRI, age 7–9 wk), which were kept in a climatized room with an artificial rhythm of dark and light periods (light from 6 PM to 6 AM), were inoculated intraperitoneally with a solution of pig-skin extract [3] or its purified fractions in saline. For in vitro labeling the animals were killed after a given time. Since the epidermal DNA synthesis exhibits a pronounced diurnal rhythm, this was done always at 9 AM. The back skin was dissected and fixed, with the epidermis beneath, on cork plates. The subcutaneous tissue was then carefully scraped off with a scalpel; traces remaining on the dermis were removed by wiping with a Kleenex towel.

Following this, the skin was cut with scissors into small pieces (2–3 mm^2) and incubated with [methyl-^{3}H]thymidine (10–15 μCi/animal) in 3 ml of Eagle's medium (with Earle's salts but without serum) at 37° C. After 45 minutes the reaction was stopped by mixing the suspension rapidly with 3 ml of an ice-cold solution containing 1 mg of unlabeled thymidine. After centrifugation the skin pieces were homogenized in 3 ml of ice water in a loosely fitting glass-Teflon homogenizer (Potter-Elvejhem). The dermal connective tissue which was not destroyed by this treatment was removed by filtration through a double layer of gauze.

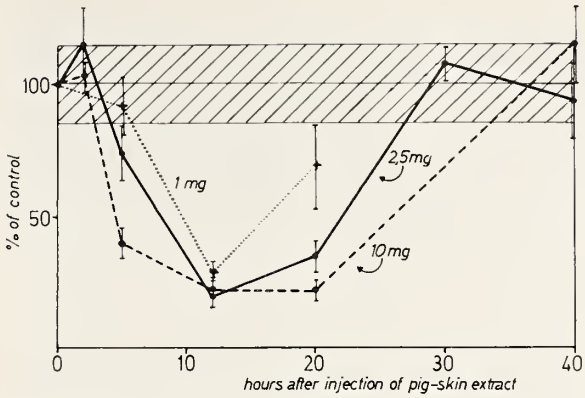
The epidermal DNA was then isolated from the filtrate by a modified Schmidt-Thannhauser procedure. For this purpose the filtrate was mixed

[3] The pig-skin extract was already partially fractionated by ethanol precipitation (*5*) and proved to contain epidermal G_2 inhibitor (chalone) in an amount corresponding to 1 chalone unit/0.4 mg. This material was generously supplied by Dr. W. Hondius-Boldingh, N.V. Organon, Oss, The Netherlands.

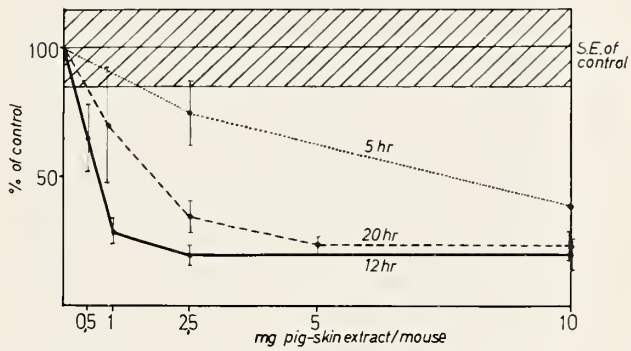
with 0.15 ml of concentrated perchloric acid and the precipitate was collected by centrifugation after standing in an ice bath for 30 minutes. After the sediment was washed 6 times with ice-cold 0.4M perchloric acid, twice with ethanol, and twice with ether, it was suspended in 1 ml of 0.3M KOH and incubated at 37° C for 3–4 hours. Protein and DNA were then precipitated with 0.06 ml of concentrated perchloric acid (1 hr.; 0° C) and washed twice with ice-cold 0.4M perchloric acid. Finally, the DNA was brought into solution by adding 1 ml of 0.5M perchloric acid and heating in a boiling water bath for 10 minutes. Portions of the supernatant were used for determination of the DNA concentration by the Burton method (*23*) and for measuring the radioactivity by liquid scintillation counting (*19*).

For in vivo experiments the thymidine was injected intraperitoneally. Then, 45 minutes later, the animals were killed and the back skin was immediately dissected, fixed on cork plates with the epidermis beneath, and chilled with liquid nitrogen. After the hypodermal tissue was removed according to the method of Sommerville and Heidelberger (*24*), the epidermal DNA was isolated as described above.

Each experiment was performed by comparing 3 experimental animals with 3 control animals which had received saline only.



TEXT-FIGURE 1.—Time course of inhibition of thymidine incorporation into DNA of mouse epidermis after injection of 1, 2.5, and 10 mg of pig-skin extract. Each *point* represents mean of at least two experiments with three control and three experimental animals each (±SE). *Hatched area* symbolizes SE of controls.



TEXT-FIGURE 2.—Dose-response relationship of inhibition of thymidine incorporation into DNA of mouse epidermis measured 5, 12, and 20 hours after injection of pig-skin extract. Each *point* represents mean of at least two experiments with three control and three experimental animals each (±SE).

RESULTS

Tissue-Specific Inhibition of Epidermal DNA Synthesis After Injection of Pig-Skin Extract

As shown in text-figures 1 and 2, intraperitoneal injections of pig-skin extracts into female mice caused a pronounced and dose-dependent inhibition of thymidine incorporation into epidermal DNA. Whereas half-maximal inhibition was observed after a single injection of approximately 0.5 mg of extract, doses larger than 1 mg led to a maximal depression of 70–80%. Depending upon the dose, the time course of the effect exhibited a lag phase of about 2–5 hours; maximal inhibition was observed between 10 and 20 hours after injection. At 30–40 hours later, the thymidine incorporation had returned to the control level.

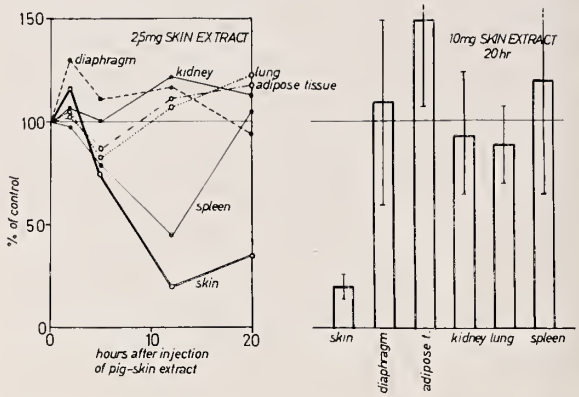
Despite the limitations mentioned above regarding the definition of a chalone, chalone-like factors must have one essential quality in common—tissue specificity of their action. This most important point cannot be stressed too strongly but, unfortunately, significant experimental evidence is very difficult to obtain. Therefore, almost all experimental approaches to the solution of this problem still carry some uncertainty. We tried to demonstrate the tissue specificity of the epidermal G_1 inhibitor in two different ways.

First, we examined the thymidine incorpora-

tion into the DNA of other tissues as compared with epidermis, after the injection of skin extract. Since we knew neither the length of the G_1 phase in those tissues nor the length of a possible lag phase between injection and the assumed inhibitory effect, we determined the labeling of DNA at different times after injection. Neither low nor high doses of pig-skin extract exhibited a significant effect on the DNA synthesis in diaphragm, kidney, lung, and adipose tissue (text-fig. 3). Only in spleen a transient but remarkable depression was observed at 12 hours after the injection, whereas after 20 hours no significant inhibition was found. However, this inhibition seems to be due to some unspecific effect, since it was observed also after injection of kidney extract or alkali-treated skin extract, both of which did not show any inhibitory effect on the epidermal DNA synthesis. These experiments were carried out by pulse-labeling in vitro; the same results were obtained, however, in control experiments performed with the in vivo technique.

As an alternative approach to investigate tissue specificity, aqueous extracts were prepared from rat liver, lung, and kidney and injected into mice in order to test their effect on the epidermal DNA synthesis. They did not inhibit at all the thymidine incorporation into epidermal DNA (text-fig. 4). Lung and kidney extracts even seemed to enhance the labeling.

Although these experiments have to be repeated with the purified epidermal G_1 inhibitor, they lend strong support to the assumption of a tissue-specific action of the factor.



TEXT-FIGURE 3.—Effect of intraperitoneal injections of 2.5 mg (*left*) and 10 mg (*right*) of pig-skin extract on thymidine incorporation into DNA of different tissues of mice. Each *point* represents mean of at least three experiments with three control and three experimental animals each. For clarity, SE are not included in the *left* text-figure.

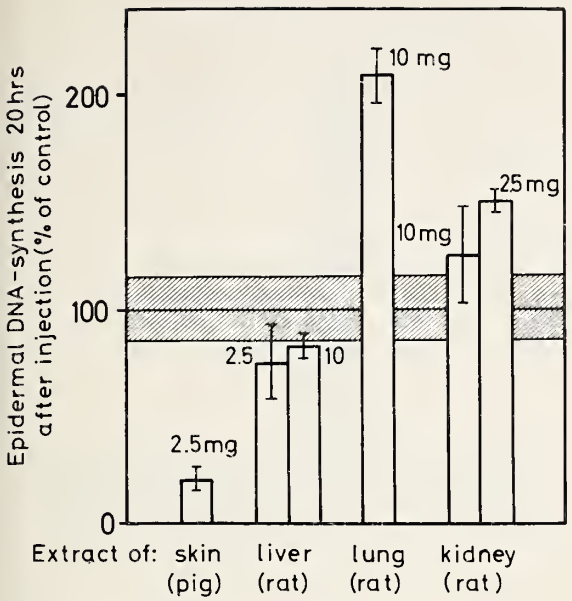
Preliminary Attempts to Characterize the Epidermal G_1 Inhibitor

The results described above do not provide any evidence on whether or not the epidermal G_1 inhibitor is identical with the epidermal G_2 inhibitor (chalone). Some experiments were therefore undertaken to characterize the G_1 inhibitor.

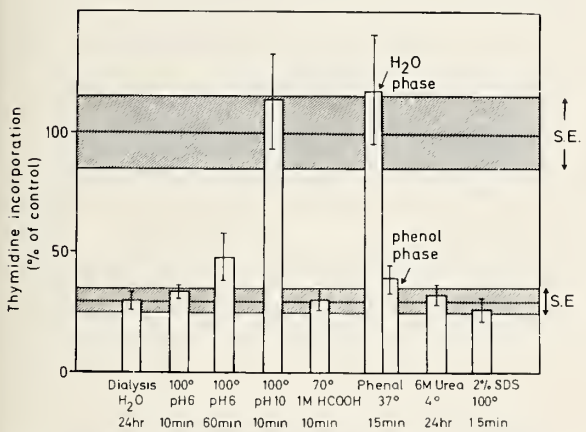
It has been repeatedly confirmed that the epidermal G_2 inhibitor is highly sensitive to heat and to proteolytic digestion (*1*, *5*). In contrast, the G_1 inhibitor could not be inactivated by heating in aqueous solution (pH 6) in a boiling water bath for 10–60 minutes or by heating in 1M formic acid at 70° C (text-fig. 5). At pH 10, however, its activity was completely destroyed. Text-figure 5 further demonstrates that the G_1 inhibitor is not dialyzable. After the pig-skin extract was shaken with 44% phenol, the active principle was completely recovered with the organic phase (which contained the bulk of proteins) whereas the aqueous phase did not exhibit any biological activity. Denaturing agents such as 6M urea (2 days; 0–4° C) or 2% sodium dodecyl sulfate (SDS) plus 5% mercaptoethanol (pH 7.2; 1–2 min; boiling water bath) did not cause any significant inactivation.

Furthermore, the G_1 inhibitor could not be destroyed by extensive digestion with trypsin, pronase, or RNase (text-fig. 6).

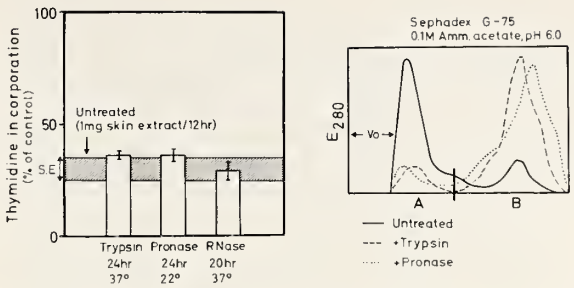
To get some information about the molecular size of the G_1 inhibitor, the pig-skin extract was fractionated on a column of Sephadex G-100 which had been previously standardized with



TEXT-FIGURE 4.—Effect of intraperitoneal injections of 2.5 and 10 mg of different tissue extracts on thymidine incorporation into DNA of mouse epidermis. Each *column* represents mean of at least two experiments with three control and three experimental animals each.



TEXT-FIGURE 5.—Effect of dialysis, heat, and treatment with phenol, 6M urea, and SDS plus mercaptoethanol on the ability of pig-skin extract to inhibit thymidine incorporation into DNA of mouse epidermis. Each experiment was carried out with 2 mg of pig-skin extract. For details, *see* text. *Hatched areas* represent mean (±SE) for control animals (*top area*) and animals which had received 2 mg of untreated pig-skin extract (*bottom area*).



TEXT-FIGURE 6.—Effect of extensive digestion with trypsin, pronase, or pancreatic RNase on ability of pig-skin extract (1 mg) to inhibit incorporation of thymidine into DNA of mouse epidermis. At least two experiments with three control and three experimental animals each were performed. The progress of the digestion was followed by the ninhydrin reaction. The text-figure on the *right* shows the elution profiles of the proteolytic digests as compared with the untreated skin extract obtained by gel filtration on Sephadex G-75. In each case the whole activity was localized in fraction A.

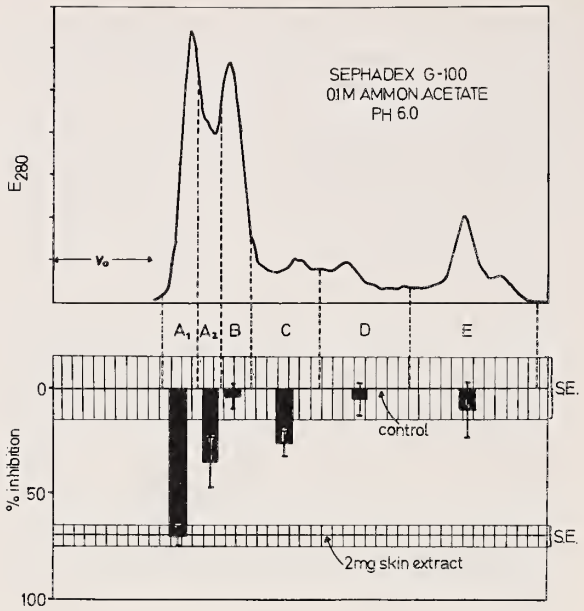
proteins of known molecular weight. The extract was resolved into at least six fractions (A_1, A_2, B, C, D, E) (text-fig. 7). The biological activity was found almost completely under the large peak (A_1, A_2) eluted with the void volume. Some activity was also found in fraction C, but this amounted only to about 10% of the total activity.

From these data we have to conclude that the epidermal G_1 inhibitor is a macromolecule with an apparent molecular weight of more than 10^5 Daltons and is characterized by a unique resistance to high temperature, denaturing agents, and proteolytic attack. We believe this to be striking evidence that the G_1 inhibitor is not identical with the epidermal chalone (G_2 inhibitor) described by Bullough and others (*1, 3–5*).

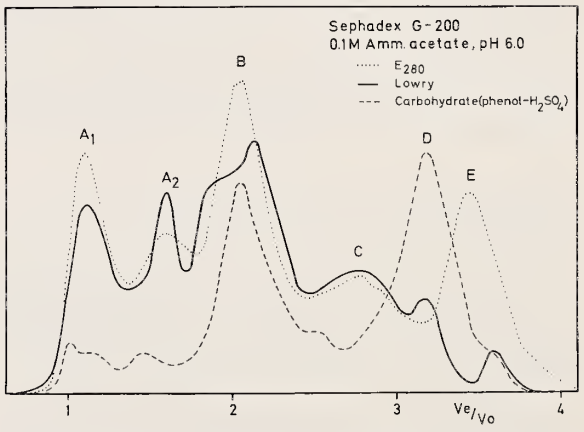
Attempts at Purification of Epidermal G_1 Inhibitor

Its resistance to proteolytic digestion as well as its solubility in phenol seemed to provide useful tools for the purification of the epidermal G_1 inhibitor.

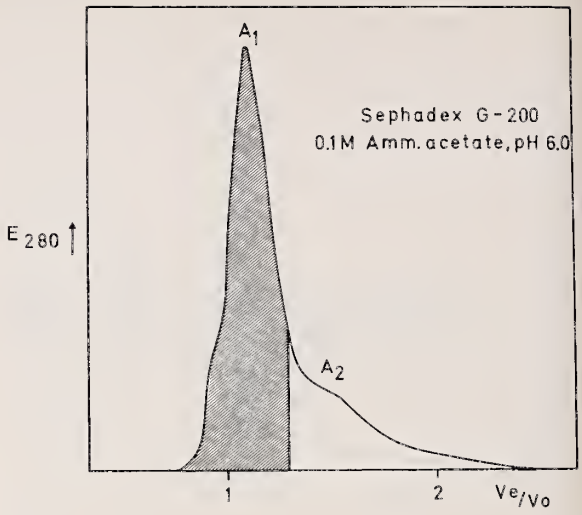
As a first step, the pig-skin extract was fractionated on a large column of Sephadex G-200 with 0.1M ammonium acetate (pH 6.0) as an eluant (0–4° C). The elution profile (text-fig. 8) demon-



TEXT-FIGURE 7.—Fractionation of pig-skin extract on Sephadex G-100 (0.1M ammonium acetate, pH 6.0, 0–4° C) and inhibition, by different fractions, of thymidine incorporation into epidermal DNA 15 hours after injection of amounts equivalent to 2 mg of unfractionated skin extract. Each *point* represents mean of at least three experiments with three control and three experimental animals each. *Hatched areas* symbolize mean (±SE) seen with control animals or with animals which had received 2 mg of unfractionated skin extract.



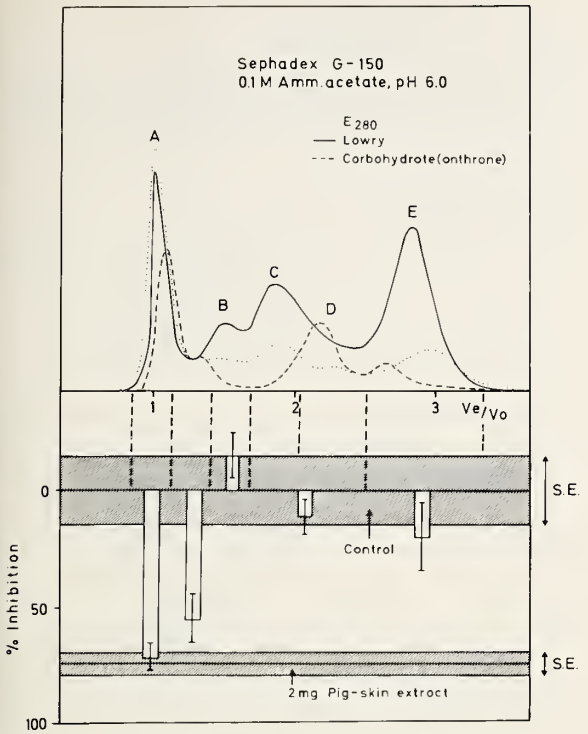
TEXT-FIGURE 8.—Fractionation of pig-skin extract on Sephadex G-200 (0.1M ammonium acetate, pH 6.0, 0–4° C).



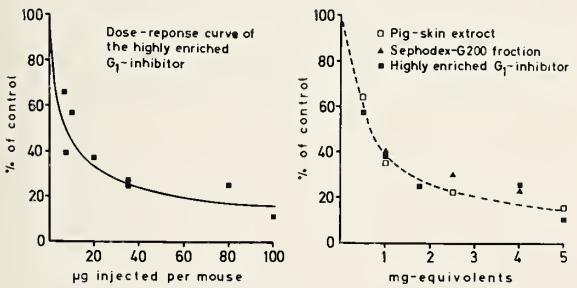
TEXT-FIGURE 9.—Rechromatography of active fraction A_1 (text-fig. 8) on Sephadex G-200.

strates that the extract consists mainly of protein and polysaccharide. The macromolecular fraction, A_1, which was eluted almost together with the void volume, contained practically all of the biological activity. It was rechromatographed on Sephadex G-200 under the same conditions (text-fig. 9).

After lyophilization, the active material was digested with 1% pronase at 30° C until the ninhydrin reaction remained almost constant (24 hr). The digest was dialyzed for 24 hours against several changes of water and then lyophilized. It still contained all of the biological activity. After gel filtration of the dialyzed digest on Sephadex G-150, the active material was eluted as a single peak practically with the void volume (text-fig. 10), indicating a molecular weight of more than 10^5 Daltons. More than 50% inhibition of the thymidine incorporation into epidermal DNA was achieved with a single injection of less than 10 μg of the active fraction (text-fig. 11). This means that, in respect to the original unfractionated lyophilized pig-skin powder (*5*), the epidermal G_1 inhibitor was about 50,000-fold enriched. Text-figure 11 indicates that the recovery of biological activity was almost complete during the purification procedure. The purified fraction was still heat stable (boiling water bath; 10 min, pH 6).



TEXT-FIGURE 10.—Fractionation of dialyzed pronase digest of active fraction (text-fig. 9) on Sephadex G-150 and inhibition, by different fractions, of thymidine incorporation into epidermal DNA 15 hours after injection of amounts equivalent to 2 mg of unfractionated skin extract. In other respects, *see* text-figure 7.

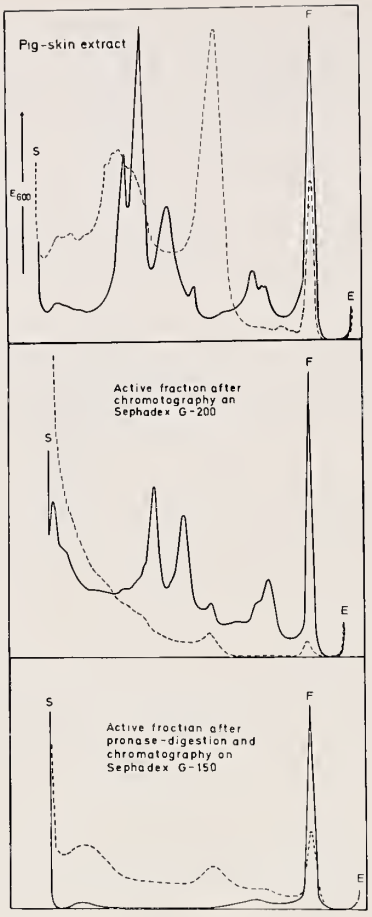


TEXT-FIGURE 11.—Dose-response relationship of inhibition of thymidine incorporation into epidermal DNA measured 15 hours after injection of active fraction A (text-fig. 10). *Right:* Dose-response relationship of effect of active fraction A_1 after Sephadex chromatography (text-fig. 8) and active fraction A after pronase digestion (text-fig. 9) compared with unfractionated pig-skin extract.

Although highly enriched, the active fraction turned out to be still heterogeneous, consisting of Lowry-positive material (protein), polysaccharide, and some orcein-positive material (? RNA). A further partial purification could be achieved by treatment with 44% aqueous phenol at 37° C. Whereas the active principle remained in the organic phase, about 80% of inactive carbohydrate material could be removed with the aqueous phase.

To check the purity of the active fraction, we used SDS-gel electrophoresis according to the method of Laemmli (*25*). To our great surprise, no material of high molecular weight absorbing at 280 nm or stainable by Coomassie Blue could be detected on the gel with the exception of two single bands migrating with or close to the front. Also, carbohydrate staining did not reveal any significant pattern. However, if the electrophoresis was carried out in the absence of SDS, a diffuse pattern of macromolecular products could be seen (text-fig. 12). Therefore we concluded that the active fraction was highly aggregated. This was confirmed by electrophoresis of the original pig-skin extract and of the active fraction A_1 obtained by chromatography on Sephadex G-200; the electrophoretic pattern obtained in the presence of SDS was considerably different from that achieved without the detergent.

Our first conclusion was that the epidermal G_1 inhibitor might have a molecular weight much lower than that indicated by gel filtration studies. We therefore repeated the fractionation of the pig-skin extract on Sephadex G-100 with 0.1–1% SDS and 1 mM mercaptoethanol in the elution buffer (0.01M sodium phosphate, pH 6.8). To be sure that intermolecular interactions were destroyed completely, the pig-skin extract was dissolved in 0.025M Tris-HCl-2%SDS-5% mercaptoethanol (pH 7.2) and heated in a boiling water bath for 1.5 minutes before putting it on the column. The chromatography was carried out at room temperature. After salts and SDS were removed by dialysis, ultrafiltration, and adsorption on Amberlite XAD-2, the fractions were tested for biological activity. The activity was found to be eluted with a relative elution volume between 1.3 and 2.2. This result leads unambiguously to



TEXT-FIGURE 12.—Densitometric patterns after polyacrylamide gel electrophoresis, in absence (------) and in presence (————) of SDS, of unfractionated pig-skin extract, fraction A_1 obtained by gel filtration on Sephadex G-200, and highly enriched fraction A after pronase digestion and gel filtration (text-fig. 10). The elcctrophoresis was carried out according to Laemmli (*25*). The gels were stained with Coomassie Blue. S = start of running gel; F = front (bromphenol blue marker); E= end of running gel.

the conclusion that the active principle is a compound of high molecular weight (probably somewhere between 1×10^5 and 3×10^5 Daltons).

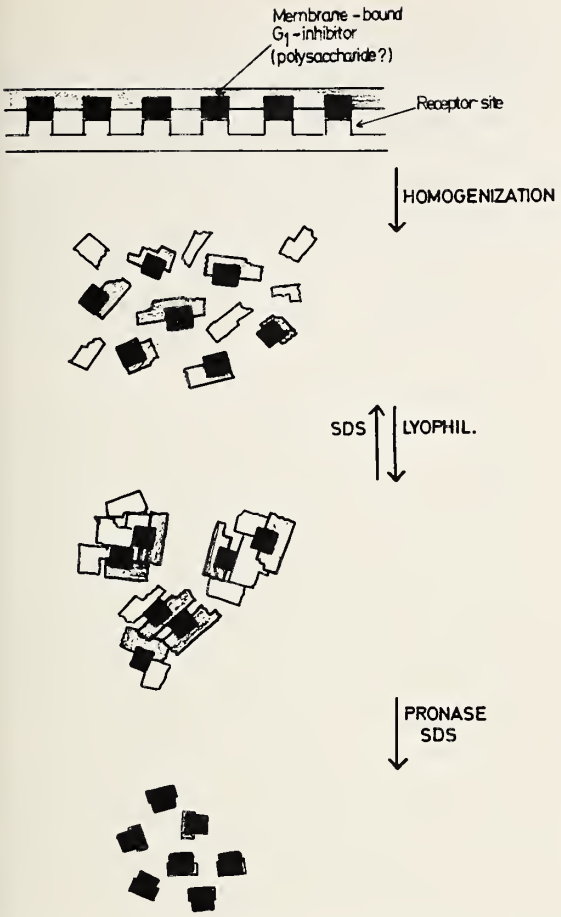
However, if the chromatography was carried out with the pronase-digested pig-skin extract under identical conditions, the biological activity was eluted mainly with an apparent molecular weight somewhere between 1×10^4 and 2×10^4 Daltons.

Thus, by proteolytic digestion, a considerable decrease of the molecular weight could be achieved without any loss of inhibitory activity. Therefore, the epidermal G_1 inhibitor as found in pig-skin extract must be a rather large molecule. According to the results described above and to preliminary analytical data, it might be a glycoprotein consisting of an inactive protein "coat," which can be removed by protease, and a rather small active "core," perhaps a polysaccharide or a pronase-resistant glycopeptide.

The elution pattern during SDS-gel chromatography gives some evidence that the protein coat may be variable in size, so that the active material as isolated from skin is not a single species with defined molecular weight but a mixture of different glycoprotein molecules all having identical active "cores."

The behavior of the active fraction during phenol extraction lends additional support to this assumption: The active principle was extracted completely into the phenol phase. However, if the extraction was carried out, under conditions such that aggregation was prevented (in the presence of SDS), the activity distributed equally between the aqueous and the organic phase. Finally, after proteolytic digestion and deaggregation by SDS, most of the activity remained in the aqueous phase. This procedure, however, caused partial inactivation of the G_1 inhibitor. The solubility of the active compound in phenol seems therefore to depend on aggregation with proteins as well as on the size of its inactive protein "coat."

We believe that our results can be interpreted most easily by the following hypothesis (text-fig. 13): In the cell, the active part of the epidermal G_1 inhibitor is bound covalently to an extended glycoprotein structure—perhaps in the plasma membrane. During maceration and homogenization this structure is broken down into small pieces, partially by mechanical forces and partially by the action of lytic enzymes. The G_1 inhibitor then goes into solution as a glycoprotein with a variable content of protein. During lyophilization the molecules become aggregated, forming large entities which are excluded even from Sephadex G-200. These aggregates can be destroyed by treatment with SDS or by high ionic



TEXT-FIGURE 13.—Tentative scheme illustrating possible events during solubilization and isolation of epidermal G_1 inhibitor. Inhibitor is thought to be a membrane-bound glycoprotein with a polysaccharide or glycopeptide as "active core" (*black rectangles*). During homogenization, the membrane structure is assumed to break down into irregular pieces which form large aggregates during lyophilization. For other details, *see* text.

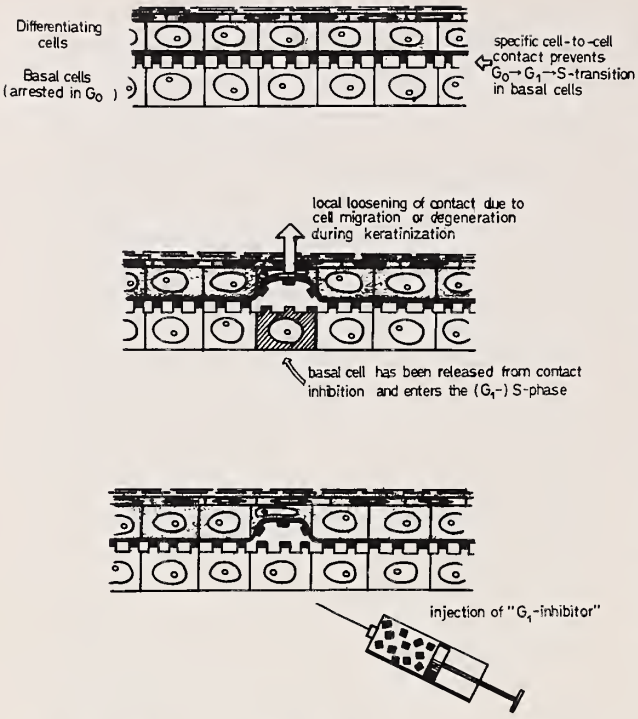
strength. In the course of the purification of the G_1 inhibitor, most of the protein will then be removed by proteolytic digestion. Finally, we should end up with the pure active "core" of the G_1 inhibitor, an approach which is now in progress in our laboratory.

DISCUSSION

There is an accumulating body of evidence to the effect that we must differentiate between at least two types of tissue-specific chalone-like regulators: those preventing a cell from entering the phase of DNA replication, by blocking in the G_1 phase, and those inhibiting the mitosis itself, by blocking in G_2. The G_2 inhibitors seem to be a rather uniform group of substances characterized by a pronounced lability to proteolytic enzymes and heat as well as by an apparently essential requirement for adrenaline. These substances have been generally assumed to be chalones in the sense described by Bullough. Regarding their mechanism of action, Bullough (*1*) has postulated that they switch off a hypothetical mitosis-operon which codes for the enzymes essential for DNA replication as well as for cell division. While a specific inhibitory action of these factors and of adrenaline on mitosis is well documented, no experimental evidence has as yet been furnished for a similar effect on DNA synthesis.

On the other hand, the occurrence of apparently tissue-specific G_1 inhibitors has been demonstrated for several tissues. As described in this paper, it has been finally shown that one and the same tissue, epidermis, obviously contains both a G_1 and a G_2 inhibitor which differ substantially from each other. Elgjo et al. (*26*) came to the same conclusion from a more indirect approach: Using the actinomycin D technique they found a considerable difference between the biological lifespans of the epidermal G_1 and epidermal G_2 inhibitors, the latter showing a much higher turnover rate.

In contrast to the adrenaline-dependent G_2 inhibitors, the G_1 inhibitors seem to be a more heterogeneous class of substances, ranging from rather small polypeptides, as described for granulocytes (*13, 14*), erythrocytes (*13*), and liver (*18*), to heat-labile proteins as found in lymphocytes (*15, 16*). Finally, the epidermal G_1 inhibitor turned out to be a heat-stable macromolecule, probably a glycoprotein consisting of an inactive protein "coat" and a rather small "active core," perhaps a glycopeptide which can be isolated after proteolytic digestion of the coat protein. This might be taken as evidence, admittedly speculative, that the factor is of membranous origin or that it is bound to a cell structure. One might imagine that for "diffuse" tissues, like blood cells and perhaps liver, a small diffusible



TEXT-FIGURE 14.—Tentative mechanism of epidermal growth control by membrane-bound inhibitor of DNA synthesis (G_1 inhibitor, *black rectangles*). For details, *see* text.

regulator is necessary (which circulates in the bloodstream) whereas in "ordered" tissues like epithelia, where each stem cell is in direct contact with a differentiated cell, the DNA synthesis is regulated by cell-to-cell contact via a membrane-bound regulatory unit.

This regulator might be developed by differentiating cells with receptor sites on the membranes of the surrounding cells, especially those of the basal layer (text-fig. 14). As long as this regulatory unit, which is thought to be identical with the epidermal G_1 inhibitor, is bound to the receptor—i.e., as long as the cells are in close contact—the basal cell will be arrested in the G_1 phase (or G_0 phase). During its maturation the differentiated cell will keratinize, migrate to the skin surface, and die. In the course of this process the contact with the underlaying basal cell will be undoubtedly weakened and lost, so that the stem cell is now released from inhibition and starts to prepare for DNA synthesis and mitosis.

This hypothesis [4] not only provides a simple explanation for epithelial growth control but also is consistent with current concepts on growth regulation in higher organisms (*27*) and with many experimental data—e.g., the results of Elgjo et al. (*28, 29*) that the epidermal G_1 inhibitor is found almost exclusively in the differentiated part of the epidermis. Furthermore, it would explain the extraordinary metabolic stability of the factor (*26*).

Whether such a membrane-bound inhibitor deserves the name "chalone" needs further investigation. One could imagine that the contact regulation of growth is a basic mechanism which is controlled by the concerted action of diffusible chalone-like inhibitors and growth-promoting factors like the epithelial growth hormone (*30*). It should be kept in mind that polypeptides regulating DNA synthesis, such as the granulocyte and liver chalones, have not yet been looked for in epidermis.

As far as the mechanism of action of chalone-like inhibitors is concerned, there is some evidence that the adenyl cyclase-cyclic AMP system is involved in G_2 inhibition, as postulated recently by Iversen (*31*). At least for epidermis, cyclic AMP and theophylline have been shown to be potent G_2 inhibitors (*32, 33*). The growth-inhibitory effect of cyclic AMP on several cell lines in vitro as well as on some tissues has been demonstrated recently in several laboratories. Finally, it has been demonstrated that epidermis contains an adenyl cyclase which is activated β-adrenergically by catecholamines and that catecholamine-dependent mitotic inhibition can be prevented by β-adrenergic blockers (*34–36*). However, the role played by the epidermal G_2 inhibitor in this mechanism is not yet clear. It is even doubtful whether, under normal physiological conditions, catecholamines are of any importance for the regulation of epidermal adenyl cyclase;

[4] If our hypothesis is true, the epidermis should contain a rather high concentration of G_1 inhibitor. Indeed, preliminary experiments have shown that 1–2 cm² of back skin epidermis of female mice contains enough inhibitor to inhibit epidermal DNA synthesis more than 50% after intraperitoneal injection (*38*).

the cyclic AMP level in mouse epidermis, as measured in vivo, varies with a pronounced diurnal rhythm, being at its maximum during the resting phase of the animal (*37*) when the adrenaline level is thought to be minimal (*1*).

Since epidermal DNA synthesis seems to be affected neither by isoproterenol nor by adrenergic blockers (*38*), an involvement of the β-receptor-adenyl cyclase system in the mechanism of action of the epidermal G_1 inhibitor may be ruled out. Very recently we have learned that, in many cell lines and tissues, regulation of cell growth as well as cell maturation and differentiation seems to depend on a critical level of intracellular cyclic AMP, which may be maintained by a permanent activation of adenyl cyclase via cell-to-cell contact [*see* for example (*27, 39–49*)]. This concept might fit very well into the working hypothesis for epidermal growth control. The reason that it has not yet been possible to demonstate any relationship between cyclic AMP and the epidermal "chalones" (G_1 and G_2 inhibitor) might be very simple: Considering the small number of epidermal cells just engaged in DNA synthesis and mitosis, a change of the epidermal cyclic AMP level by loss of cell-to-cell contact would be only local and probably not measurable relative to the overall level of the nucleotide.

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Commentary on "A Tissue-Specific Factor Inhibiting DNA Synthesis in Mouse Epidermis" by F. Marks [1]

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THE WORK of Hondius Boldingh and Laurence (*1*) and the present paper by Dr. Marks clearly indicate that epidermal chalones are glycoproteins. Independently of epidermal chalone research, a considerable effort has been made to characterize glycoproteins of the skin, but little cross-fertilization between researchers in the field of epidermal chalones and glycoprotein chemistry has occurred. The task of purifying epidermal chalones may be facilitated by the application of well-established techniques for the isolation of epidermal glycoproteins.

Most glycoproteins of the dermis are of the mucopolysaccharide type. Thus, hyaluronic acid, dermatan sulfate, chondroitin sulfates, heparin, heparin sulfate, and keratan sulfate have been isolated from the skin and shown to be of dermal origin (*2–4*). Little is known about the epidermal glycoproteins. A recent report by Shimada et al. (*5*) indicates that epidermal glycoproteins are similar to epithelial secreted and surface-type glycoproteins. They in fact contain L-fucose, D-hexosamines, D-galactose, D-mannose, D-*N*-acetylneuraminic acid (NANA), and, of course, like the mucopolysaccharides, a covalently linked protein moiety.

Both classes of compounds contain D-hexosamines; hence, these cannot be used as a distinctive feature of either one. All acidic mucopolysaccharides, however, contain uronic acids, constituting approximately 50% of their molecule, except for keratan sulfate, a polymer of *N*-acetyl-D-glucosamine and D-galactose. Hence, uronic acid may be used as a distinctive characteristic of acidic mucopolysaccharides. The epidermal glycoproteins are of the blood group substance type, and they contain L-fucose and NANA as their terminal sugars and D-mannose, D-galactose, and hexosamines as their most prominent components. Either L-fucose or D-NANA may therefore be considered to be their distinctive characteristic.

This second class of glycoproteins has been found to be altered after virally induced transformation (*6*). It appears that, after transformation, these molecules contain different amounts of their chemical constituents, compared to normal conditions. Moreover, new glycoprotein antigens, characteristic of the embryonic state, appear after transformation—i.e., carcinoembryonic antigen and α-fetoprotein (*7, 8*). There is a growing body of evidence to show that glycoproteins control cell division and social-cellular behavior and that drastic changes occur in cellular behavior when plant agglutinins are used to mask surface glycoproteins (*9, 10*). It also has been shown that these agglutinins specifically interact with the sugar moieties of glycoproteins on the cell surface.

Another interesting biological function has been attributed to glycoproteins: that of messenger molecules for epithelial mesenchymal interactions. In this function, acidic mucopolysaccharides synthesized in the mesenchyme seem to play a role (*11, 12*). Since the definition of chalone requires that this substance be produced by the same tissue that it controls and since reports

[1] Presented at the First Symposium of the International Chalone Conference, Brook Lodge, Augusta, Mich., June 5–7, 1972.

[2] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

have appeared that heparin, as well as other mucopolysaccharides, may slow down cell division (*12*), it seems crucial to establish whether the chalone glycoprotein is of epithelial or mesenchymal origin. A method has recently been published (*5*) to accomplish separation of these two classes of macromolecules in rabbit skin by an extraction with the detergent cetylpyridinium chloride (CPC). The acidic mucopolysaccharides are precipitated as a complex with CPC. The epithelial glycoproteins are left in the supernatant fraction. This can be monitored by uronic acid. No uronic acid-containing material is present in the supernatant.

Once it is established that either one or the other of these classes of molecules has the chalone activity, then purification can be aided by the use of specific labeled precursors. One can label glycoproteins specifically by using, in vivo or in vitro, labeled L-fucose. A double-label experiment with ^{3}H-fucose and ^{14}C-labeled amino acid will afford labeling of both moieties of the glycoprotein molecule. The ratio of $^{3}H/^{14}C$ should increase during purification, to reach constancy when a pure chalone glycoprotein has been obtained.

Marks has reported that pronase and trypsin treatment do not affect the G_1 chalone activity. Hence, to increase the yield of G_1 chalone, one may remove the extraneous proteins by proteolytic digestion of the epidermis, precipitate the mucopolysaccharides by CPC, then precipitate the G_1 chalone by 70% ethanol, and finally purify it by ion exchange chromatography and gel filtration. We hope that these suggestions may facilitate the purification of G_1 and G_2 chalones.

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Epidermal Chalone and Inhibition of DNA Synthesis [1]

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SUMMARY—Aqueous epidermal extracts from newborn and adult mouse epidermis were partially purified by alcohol and ammonium sulfate precipitation to give fractions active in inhibiting DNA synthesis in cultured mouse skin. The active protein fraction consisted of at least two components having a molecular weight between 61,000 and 70,000 Daltons. A heat-stable dialyzable component, separated from the epidermal extract prior to or after purification, was active in inhibiting DNA synthesis. The newborn mouse extract has a component in the dialysate, which is not present in the adult mouse extract, which activates the inhibition of DNA synthesis with or without the protein fraction (F_1 and F_2). Epinephrine was not essential for maximal inhibition in all fractions although, in some, inhibition of DNA synthesis was enhanced by the addition of epinephrine. It has not been demonstrated at this time whether the epidermal chalone which inhibits mitosis is the same as or different from that which inhibits DNA synthesis. Preliminary evidence suggests that they may be different substances.—Natl Cancer Inst Monogr 38: 93–98, 1973.

RENEWAL OF interest in chalone occurred with the finding by Bullough and Laurence (*1–3*) that an aqueous extract of epidermis (from adult mice, rats, guinea pigs, and rabbits) inhibits epidermal cellular mitosis. The aqueous epidermal extract was shown to be specific for epidermal tissue and did not inhibit mitosis in other tissues. Aqueous extracts from nonepidermal tissue failed to inhibit mitosis in epidermal tissue. The action of an aqueous extract (chalone) of rat epidermis on DNA synthesis was examined (*4*), and it was found to inhibit only epidermal mitosis. Subsequent workers have found that extracts of stratum corneum (*5*) or skin (*6*) injected intraperitoneally inhibit DNA synthesis in the epidermis. Delayed inhibition of DNA synthesis was demonstrated to occur only after incubations for 9–12 hours. Purified pig-skin extracts have also been shown to exert a tissue-specific inhibition of epidermal DNA synthesis (*7*).

The cellular processes responsible for the control of mitosis and DNA synthesis in the normal and abnormal epidermis have been a major interest in my laboratory. The main stimulus for this interest is the need to explain our initial observations that an accelerated turnover occurs in the psoriatic epidermis (*8*). A simplified mechanism or a model system was sought that would explain the stimulation or lack of inhibition of the epidermal proliferative processes in the psoriatic epidermis.

The developing epidermis of the chick embryo skin exhibits an accelerated proliferation in the 12-day embryo and a rapid decrease in proliferation in the 21-day embryo (*9, 10*). One explanation for the changes at day 12 is a simultaneous stimulation of both DNA synthesis and mitotic activity. The decrease from peak stimulatory activity after day 12 may be attributed to decreas-

[1] Presented at the First Symposium of the International Chalone Conference, Brook Lodge, Augusta, Mich., June 5–7, 1972.

ing levels of stimulation or neutralization by new synthesis of epidermal inhibitor which, by day 21, is fully active and assumes its regulatory role.

It has been shown (*11*) that the 14-day-old epidermis is inhibited upon the addition of 21-day chick embryo skins or embryo extract suspension or supernatant to the growth medium. When 12-day chick embryo skin, skin suspension, or embryo extract suspension is added to the growth medium, it stimulates DNA synthesis in 16- and 17-day chick embryo skin.

These experiments suggest a regulation of DNA synthesis by an inhibitory substance from the older tissue, which may act in a manner similar to the mode of action of epidermal chalone. The gradual appearance of chalone in the aging embryonic skin suggests its possible function thereafter in the maintenance of homeostasis in the normal proliferating epidermis.

To clarify the role of chalone in epidermal proliferation and to understand how it is involved in DNA synthesis, the chalone was isolated from newborn mouse and cow snout epidermis (*12*). Each of these extracts was found to inhibit DNA synthesis in both the epidermis and dermis of newborn mouse and chick embryo skin. Cow snout aqueous epidermal extract was stable in incubations for 2, 4, 6, and 8 hours and inhibited DNA synthesis for these periods. To obtain maximal inhibition in the dermis the extract required the addition of epinephrine; in the epidermis, both epinephrine and hydrocortisone had to be added to the extract.

With the observations that an aqueous epidermal extract is active in inhibiting thymidine incorporation into DNA in our skin culture system, purification of the extract was undertaken.

METHODS

Epidermis was obtained from newborn mice (*hr/hr, hr/+*), 1–3 days old. Each mouse was immersed in water at 55° C for 30 seconds and immediately placed on ice (*12*).[2] The epidermis was then carefully dissected (or peeled) from the dermis. This epidermal envelope was immediately lyophilized to dryness and stored at −20° C until used.

Several hundred epidermal envelopes, dry weight 3.22 g, were frozen by immersion in liquid nitrogen and ground with alumina (type 305) in a mortar. The resulting paste was collected and centrifuged at 27,000 × *g* for 30 minutes to separate the clear supernatant fraction (S_1) from the tissue residue. Supernatant protein was determined by measuring of the 280/260 ratio (Zeiss spectrophotometer PMQ II) and calculating the amount of protein from nomograph (E. Adams).

Possible bacterial contaminants were removed from the supernatant by passing it through a Millipore filter (0.45μ). The volume was then reduced by lyophilization, and samples were removed prior to alcohol fractionation. Absolute ethanol was added to the supernatant, and precipitates were collected between 0 and 33%, 33 and 70%, and 70 and 80%. No additional precipitate resulted after 80% alcohol. The alcohol and water were removed by lyophilizing to dryness. Fractions were labeled as indicated in table 1. After determination that fraction F_4 was active, this fraction was further fractionated by ammonium sulfate precipitation. The only precipitate obtained was at 60% saturation. The ammonium sulfate was removed from both the 60% fraction and the 100% supernatant by exhaustive dialysis against water.

Samples of S_1, which were lyophilized to dryness and stored at −20° C, were further processed to recover the dialyzable components and the protein remaining in the sac.

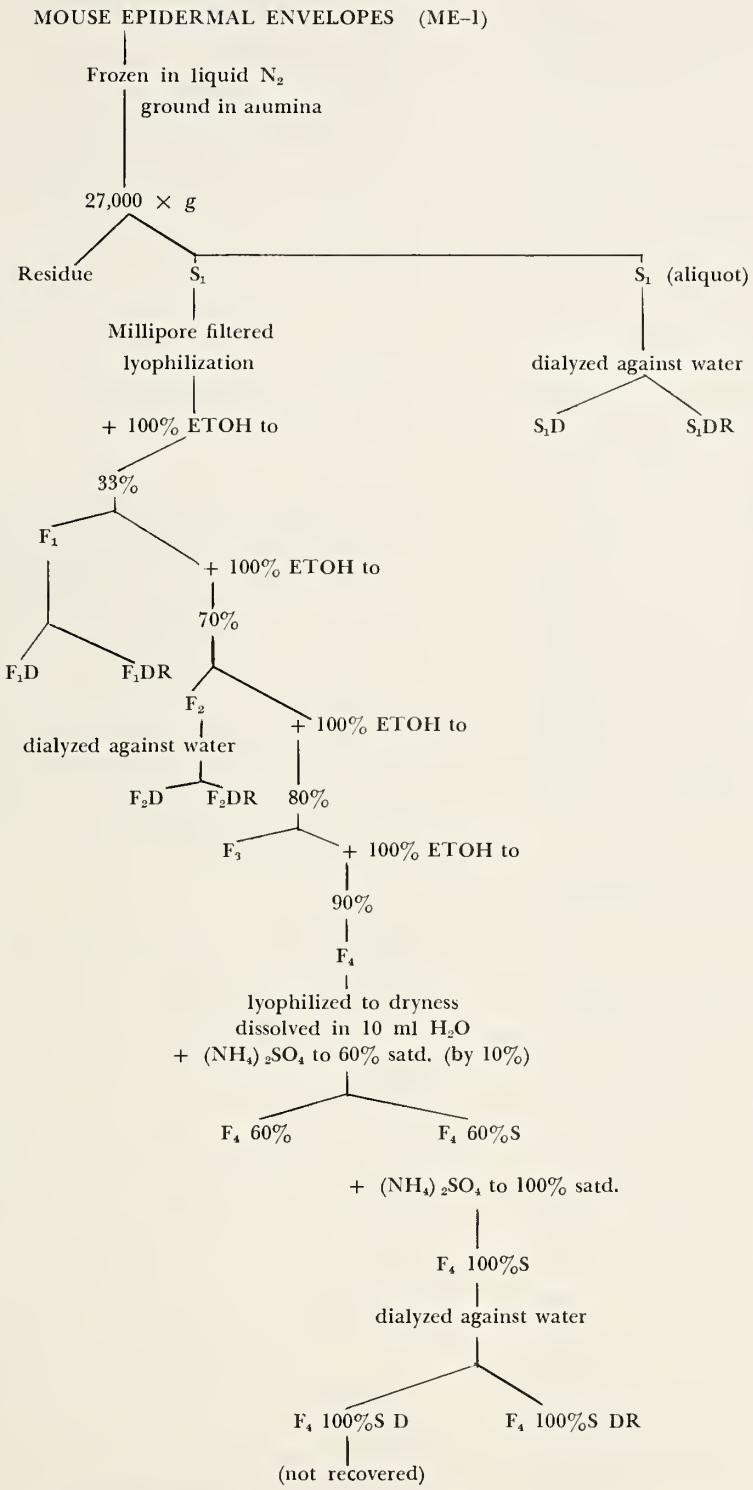
Epidermis of mice (*hr/hr*) 2–10 months old was obtained in a similar manner except that the skin was dipped for 45 seconds at 55° C. The epidermis was then processed as before except that no ammonium sulfate fractions were prepared from the F_4 fraction of the adult epidermal extract.

Fractions obtained from the epidermal extracts were tested by measuring their action on thymidine-6-^{3}H incorporation into DNA of cultured mouse skin.

The skin for culture was obtained from female hairless mice (*hr/hr*) 28–50 days old. Prior to dissection of the skin from the ether-terminated mouse, the skin was well cleaned with an alcohol

[2] Appreciation is expressed to Lawrence J. Field for his help in obtaining the epidermal envelopes.

TABLE 1.—*Procedure for isolation*



swab. Sections of about 1 cm^2 were layered on siliconized lens paper and floated in BGJ-M medium on ice until cultured.

The skin was cultured as described previously (*13*). To the BGJ-M culture fluid (*14*) was added different concentrations (25–500 μg/ml) of the fraction to be tested, epinephrine bitartrate, 2×10^{-5}M, and 3.3 μCi of thymidine-6-^{3}H (specific activity, 6.5 Ci/mmole, New England Nuclear).

After addition of the skin and medium to the culture dishes, they were placed in an air-tight Lucite box and aerated for 3–4 minutes with a mixture of 5% CO_2 and 95% air. The sealed box was then incubated at 37° C for 4-hour periods. The incubations were terminated by immersing the tissues in cold 10% trichloroacetic acid (TCA). This TCA was removed and fresh TCA was added prior to overnight refrigeration.

DNA was isolated by a modification (*15*) of the Schmidt-Thannhauser procedure. Of the 1.2 ml obtained by hot extraction (90° C) with 5% TCA, 0.35-ml portions were used for duplicate assays of DNA by the diphenylamine reaction of Burton (*16*). The radioactivity was also measured in duplicate (0.1-ml aliquots) in toluene-Cellosolve scintillation fluid (PPO-POPOP) in a Packard automatic Tri-Carb liquid scintillation spectrometer system.

Specific activity was calculated as total counts per minute (cpm) in the isolated DNA per absorbance unit.

In some experiments the specific activity was expressed as total cpm per milligram dry weight of tissue. This tissue was washed extensively with cold TCA and then with distilled water. The tissue was then dried by lyophilization for 4 hours and weighed. Repeated lyophilization did not change the final weight. The tissue was prepared for counting in the liquid scintillation system by solubilizing in 1 ml of Protosol (New England Nuclear) overnight and counting each sample as described above.

The fraction, $F_4$100%SDR, and the starting extract supernatant, S_1 of newborn and adult epidermis (NB and A), were analyzed by disc electrophoresis (*17*).[3] The samples were run on 7% polyacrylamide gel in 1% SDS at pH 8.0 for 90 minutes at 8 mA per tube. The components were stained in Coomassie Blue and destained by extraction in 10% acetic acid.

RESULTS

Prior to fractionation of the aqueous extract, S_1 was added to the skin culture system in order to measure its influence in inhibiting thymidine incorporation into DNA. The separate actions of the epidermal extracts from newborn and adult mouse in inhibiting the incorporation of thymidine into DNA are indicated in tables 2 and 3. Inhibition by newborn mouse S_1 was enhanced by the addition of epinephrine. The adult epidermal S_1 was able to inhibit maximally without the addition of epinephrine.

TABLE 2.—*Action of newborn mouse epidermal aqueous extract fractions on thymidine-6-^{3}H incorporation into DNA in mouse skin*

Fraction	Inhibition*	
	Without epinephrine	With epinephrine
S_1	+	++
S_1DR	++	++
S_1D	++	++
F_1	+	+
F_1DR	0	—
F_2	++	++
F_2DR	0	—
F_3	+	+
F_4	+	++
$F_4$60%	0	0
$F_4$100%S	++	++
$F_4$100%SDR	+	++

*+ = 10-25%; ++ = 25-50%.

Most alcohol fractions of newborn mouse epidermis were inhibitory. After dialysis however, fractions F_1 and F_2 were no longer inhibitory. F_1 and F_2 from the adult mouse were inactive prior to removal of the dialyzable component. The dialyzable components were obtained from an extract sample that had been put aside and was prepared after the fractionation of the major portion of the extract. It is therefore unknown whether the activities found for the undialyzed fractions are attributable to

[3] Run by Dr. Keith Shelton, Department of Biochemistry, Medical College of Virginia, Virginia Commonwealth University, Richmond, Va.

TABLE 3.—*Action of adult mouse epidermal aqueous extract fractions on thymidine-6-^3H incorporation into DNA in mouse skin*

Fraction	Inhibition*	
	Without epinephine	With epinephine
S_1	++	++
S_1DR	+	++
S_1D	+	+
F_1	0	0
F_2	0	0
F_3	++	++
F_4	++	++
F_4DR	+	++
F_4D	+	+
F_4D (b)	++	++

*+ = 10–25%; ++ = 25–50%.

the protein component, the dialyzable component, or the presence of both components. Fraction F_1 was the largest fraction in both newborn and adult mouse epidermis.

Also inactive prior to dialysis was newborn extract $F_4$60%. After dialysis, $F_4$100%SDR was active, and its activity was enhanced by epinephrine. Before dialysis, $F_4$100%S did not require epinephrine for maximal inhibitory action. The question of whether epinephrine is one of the components of the dialysate was unanswered, but the dialyzable component has a maximum at 274 nm which is different from that of epinephrine.

The dialyzable component was found to be more than one compound. Preliminary resolution of the dialyzable component by high-voltage electrophoresis indicates that more than 10 components are present. None of these components corresponds to uridine or thymidine intermediates.

Experiments with fraction F_4D(b) from adult mouse extract indicated that the dialyzable component is stable after boiling for 15 minutes. In fact, the inhibitory activity was slightly enhanced.

Some of the fractions (S_1, F_4, and $F_4$100%SDR of the newborn and S_1DR and F_4DR of the adult) showed enhancement of their inhibition by the addition of epinephrine.

Disc electrophoresis was carried out for fraction $F_4$100%SDR and compared to the original extract supernatant, S_1 (both newborn and adult). The S_1 fractions showed many similarities in the approximately 13 components. The purified fraction, $F_4$100%SDR, had two components with molecular weights ranging from 61,000 to 70,000 Daltons.

DISCUSSION

The results suggest that several proteins in the aqueous epidermal extract, alone or in conjunction with some compounds in the dialyzable component, can inhibit thymidine incorporation into DNA. This is supported by the findings that all the alcohol fractions, prior to dialysis, from the newborn mouse extract inhibit DNA synthesis except for the 60% ammonium sulfate precipitate of fraction 4. Fraction 3, which is active in both newborn and adult mouse extracts, is the 70–80% fraction used originally by Bullough and Laurence (*18*) and further purified by Hondius Boldingh and Laurence (*19*) from pig skin. They (*19*) obtained a partially purified chalone preparation which contained hydroxyproline, a dermal constituent.

It is uncertain what contribution these dermal components make to the tissue-specific mitotic inhibition observed and whether dermal mitotic or DNA inhibition (*7*) is influenced by any of the constituents of pig skin extract. It therefore seems essential that the tissue to be used for the preparation of extracts be epidermal, so that the results then can be related more directly to the inhibition of epidermal mitosis, epidermal DNA synthesis, or tissue specificity.

Although the factor inhibiting DNA synthesis could be the same as that which inhibits mitosis, it is possible that they are different substances. This is supported by the disc electrophoresis pattern for the most purified fraction, $F_4$100%SDR. The patterns suggest that this fraction has at least two components which have molecular weights in the range of 61,000–70,000 Daltons. The active fraction of Hondius Boldingh and Laurence, although contaminated with hydroxyproline, has a molecular weight of 30,000–40,000 Daltons.

Besides the active protein fractions, the dialyzable component of the epidermal extract acts independently or in conjunction with the protein fractions obtained from the extract to inhibit

the incorporation of thymidine-6-^{3}H into DNA synthesized in the mouse skin. Preliminary work indicates that the dialyzable component contains material with ultraviolet absorption characteristic of nucleotides. The absence of uridine and thymidine intermediates from this dialyzable component rules out the possibility that these components contribute to the observed inhibition by isotope dilution. When this fraction was boiled for 15 minutes, the absorption maximum and minimum shift but the fraction is still active in inhibiting DNA synthesis.

Although epinephrine is active in enhancing inhibition by some of the fractions, the specific mechanism of how the fractions alone or in conjunction with epinephrine inhibit DNA synthesis is unknown. It has been suggested (*20*) that the inhibition of mitosis results from action of the chalone at G_2 while the inhibition of DNA synthesis results from action at G_1 or S.

The previous observations (*12*) that epidermal extract is not tissue-specific and inhibits DNA synthesis in the dermis also suggests that the factor or factors which inhibit DNA synthesis are different from those which inhibit epidermal mitosis. Epidermal and skin extracts have been reported by others to be tissue-specific and to inhibit mitosis only in the epidermis.

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The Epidermal Chalone Mechanism [1]

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IN ADULT MAMMALS, tissue mass remains remarkably constant, at least until the onset of old age. Most tissues are mitotic and therefore the constancy of their mass must depend on closely controlled rates of cell gain and cell loss as well as on an ability to regenerate after abnormal cell loss by trauma. Some 10 years ago, and with special reference to conditions in the epidermis, it was proposed that tissue mass may be limited by a simple antimitotic negative-feedback mechanism. This was done for two main reasons: first, to satisfy Occam, it was the simplest theory that could account for the facts as they were then known (*1–3*); and second, because it had been discovered that in epidermis there indeed exists a tissue-specific antimitotic messenger molecule, later called the "epidermal chalone" (*4*), of the exact type needed in such a feedback mechanism (*5–7*).

Since then, more than a dozen different tissue-specific chalone systems have been recognized (*8*), and there has been a general but cautious acceptance of the negative-feedback mitotic control theory. It now seems certain that all mitotic tissues will prove to contain chalone systems and that a new and highly important family of chemical messengers has been discovered.

Recently, however, more detailed analyses have shown that chalone systems are more complex than was originally thought (*9*), and the present paper gives an up-to-date assessment of the epidermal control mechanism.

[1] Presented at the First Symposium of the International Chalone Conference, Brook Lodge, Augusta, Mich., June 5–7, 1972.

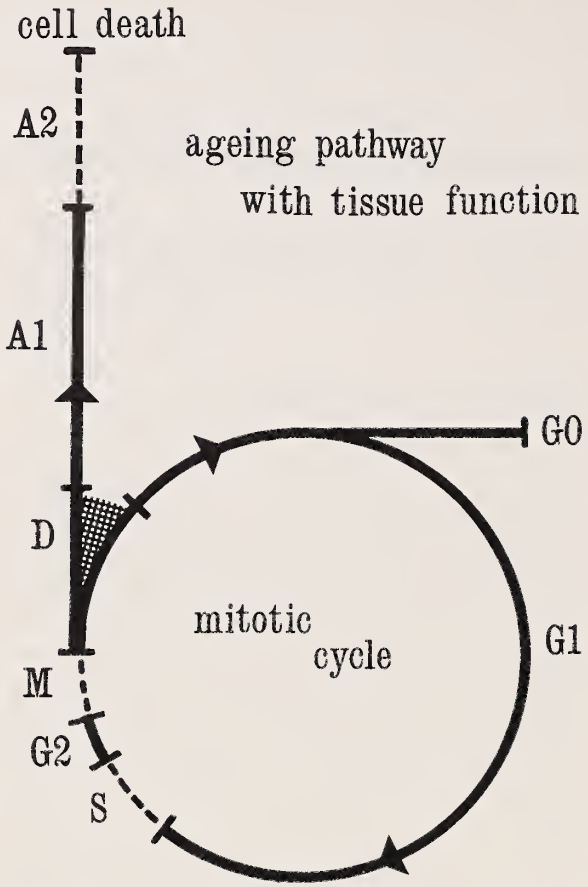
THE MITOTIC CYCLE AND THE AGING PATHWAY

In its passage from mitosis to mitosis and to its death with keratinization, an epidermal cell follows the route shown in text-figure 1, and this diagram probably also applies to all other mitotic tissues. In the basal epidermal layer there are cells in all phases of the mitotic cycle. This cycle may proceed so rapidly that it is completed within 24 hours or so slowly that, with no cycle discernible, the cells are considered to be static in G_0. Not all the basal cells, even if adjacent to each other, move around the cycle at the same speed.

Present evidence suggests that the epidermal chalone, together with the stress hormones (*6, 10*), acts to slow the whole mitotic cycle with the exception of the phases of DNA duplication and of mitosis. Both these phases are all-or-none reactions, since the necessary syntheses are completed in advance. Thus the chalone acts to prevent the commencement of these phases, and chalone action has been most conveniently assayed in terms of inhibition of S phase or mitosis.

After the completion of a mitosis, the two daughter cells enter the dichophase (*11*) in which the decision is made whether to reenter the mitotic cycle or to become postmitotic and to prepare for tissue function. The manner in which this decision is made is discussed below; it evidently involves the activation of one or another alternative genetic program. If those genes are activated that specify the enzymes that drive the mitotic cycle, then this whole cycle must pass to completion. It is an all-or-none decision.

If, alternatively, a cell becomes postmitotic, it



TEXT-FIGURE 1.—The course of cell life in epidermis. In the dichophase, D, cells emerging from mitosis choose between entry into G_1 (an all-or-none decision) or into A_1 (a potentially reversible decision).

enters the aging pathway and passes through two phases, A_1 and A_2 (*12*). In A_1 (e.g., stratum spinosum) the genes for cell aging and for keratin precursor synthesis are activated, while the mitotic genes are suppressed. This is not an all-or-none decision. After epidermal damage, when the intracellular chalone concentration may be decreased by about 50% (*13*), an A_1 cell can revert to mitosis.

In A_2 (e.g., stratum granulosum) such reversion is no longer possible. The evidence (*12*) suggests that all the genes have been finally silenced, that the nucleus is degenerating (*14*), and that the cell is surviving only by the activity of preformed messenger RNA.

All postmitotic cells have an average life expectancy that is characteristic of the tissue. In normal epidermis this may be about 7–21 days, depending on the species and on the region of the body (*15, 16*). However, the value is not constant; it varies in relation to the mitotic rate. Thus, with very low mitotic activity (as during continuous stress), it may be 2 or 3 months (*17*); in normal epidermis it is about 2 or 3 weeks; and with high mitotic activity (as in psoriasis), it may be decreased to only about 3 or 4 days (*18, 19*). Clearly, postmitotic aging is not a passive process. It is controlled by some aging mechanism which in the epidermis is inhibited by the epidermal chalone (and by the cooperating stress hormones).

TISSUE FUNCTION

The processes of postmitotic cell aging and of tissue function, although normally accompanying each other, are at least semi-independent. Tissue function may occur in A_1, as in kidney and liver (the cells of which readily revert to mitosis if the chalone concentration falls too low); it may occur in A_2, as in erythrocytes and granulocytes; or it may even occur after death, as in epidermis and other external tissues.

If function occurs in A_1, then the tissue is typified by a low mitotic rate and a low rate of cell death; in other words, a high chalone concentration holds the cells almost static in A_1. If function occurs in A_2 or in dead cells, then the tissue is typified by a relatively high mitotic rate and a relatively high rate of cell death; in other words, a lower chalone concentration permits active mitosis and a relatively fast passage of the cells through A_1.

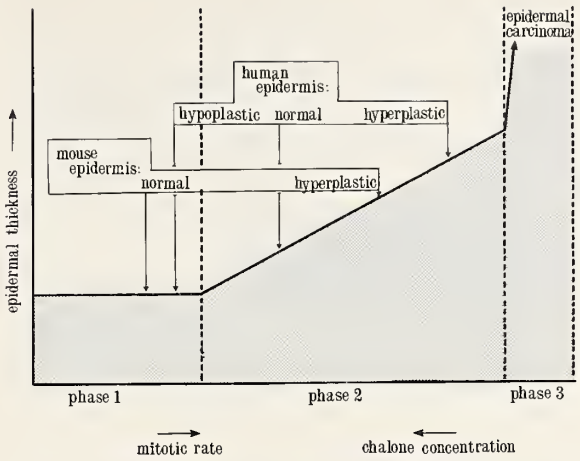
Further evidence comes from undifferentiated carcinomas in which the cells have lost their ability to function. Such cells, when they become postmitotic, are still able to age and die in an apparently normal manner (*20*). Thus the choice made in a dichophase cell is between mitosis and aging and not between mitosis and tissue function. Postmitotic aging may prove to be a gene-controlled program.

MITOTIC ACTIVITY AND EPIDERMAL THICKNESS

The negative-feedback theory of mitotic control necessarily proposes that increased mitotic activity must lead to increased tissue mass. However, a study of mouse epidermis (*9*) has shown that, within wide limits, changes in chalone concentration (or in stress hormone concentration) leading to changes in the mitotic rate do not cause changes in the epidermal thickness or mass. Increases or decreases in the mitotic rate are so precisely matched by similar increases or decreases in the rate of postmitotic cell aging that the ratio of the rates is constant: rate (mitosis): rate(aging) = K. This fact alone disposes of the theory that epidermal mass is controlled in a simple manner by a negative-feedback mechanism.

However, in other types of epidermis the situation is different. In man, pig, and guinea pig, any change in the epidermal mitotic rate is accompanied by a parallel change in the epidermal thickness, even though, as in mouse epidermis, it is also accompanied by a parallel change in the rate of postmitotic cell aging (*9*).

When the whole possible range of epidermal mitotic activity, from very low to very high, is considered and is compared with epidermal thickness, the relationships shown in text-figure 2 are disclosed. In phase 1 (e.g., mouse epidermis), characterized by a lower mitotic rate, changes in the rate of cell production do not result in changes in epidermal thickness; in phase 2 (e.g., human epidermis), characterized by a higher mitotic rate, changes in the rate of cell production do result in changes in epidermal thickness; and in phase 3 (e.g., rapidly growing epidermal carcinomas), characterized by the highest mitotic rate, there is an explosive increase in epidermal mass. These phases are examined separately below.



TEXT-FIGURE 2.—Relationships between epidermal mitotic rate and epidermal thickness.

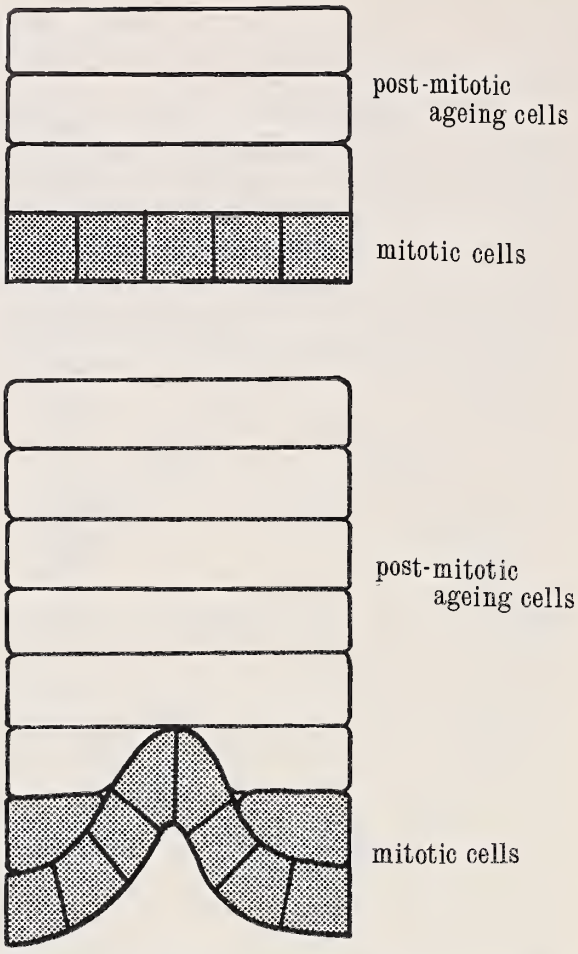
Phase-I Type Epidermis

The normal epidermis of mouse and rat is relatively thin. It has a relatively low mitotic rate, a relatively slow rate of postmitotic cell aging, and a flat junction with the underlying dermis (text-fig. 3, *upper*). Each mitosis is usually so oriented that both daughter cells remain in the basal layer (*7*); it is some adjacent cell that is pushed out. Recent information from Christophers (*21*) has shown that the extruded cell is postmitotic, since it contains keratin precursors that can be diagnostically stained. Thus the basal layer contains both mitotic and postmitotic cells [*see also* Iversen et al. (*22*)], and the implication is that the latter have a weaker grip on the dermis.

The thickness of phase 1 epidermis remains constant because, with more mitoses, more postmitotic cells are pushed from the basal layer to age more quickly; with fewer mitoses, fewer postmitotic cells are pushed out to age more slowly. A similar situation exists in mouse sebaceous glands (*17*) and probably also in all tissues that have a relatively low mitotic rate.

Thus, in phase 1, tissue mass is determined first by the relatively high chalone concentration that places the tissue in that phase and second by the time taken for the postmitotic cells to age and die.

The time taken for such cells to age and die is characteristic for each tissue and, in the epidermis, for each epidermal region. In mouse, ear epidermis is much thicker than dorsal epidermis although the mitotic rates are approximately the same. This is because the rate of



TEXT-FIGURE 3.—Structure of phase-1 type (*upper*) and phase-2 type epidermis (*lower*).

postmitotic cell aging is characteristically slower in the ear epidermis. Thus inherent differences in tissue mass are related to inherent differences in the aging rates of the postmitotic cells.

It is important to note that, although the mitotic rate and the aging rate normally change in unison, they can be separately influenced. Thus, when rat sebaceous glands are treated with estrogens, the mitotic rate remains unchanged, the rate of postmitotic cell aging is accelerated, and the glands shrink in mass (*23, 24*).

Phase-2 Type Epidermis

In phase 1, an increasing mitotic rate involves not only an increasing speed of mitotic cycle but also an increasing number, or proportion, of basal cells that are involved in the mitotic cycle and a corresponding decreasing number, or proportion, of basal cells that are able to become postmitotic. The breakpoint into phase 2 comes when all the basal cells are in the mitotic cycle and when, consequently, no postmitotic cells are available to be pushed out. From this point onward, each mitosis causes an increase in the lateral pressure in the basal layer, which therefore begins to fold (or, as in the mouse, to double).

Normal human epidermis is in this folded condition so that any change in the mitotic rate results in a change in the degree of folding (text-fig. 3, *lower*). The more the basal layer folds (or doubles) the greater is the number of mitotic cells per unit area of skin and, since the number of postmitotic cells maintains an apparently constant relation to the number of mitotic cells, the greater the degree of folding the greater the number of distal cells and the thicker the epidermis. In man, a high degree of basal folding is found in such hyperplastic conditions as psoriasis and the various types of benign epidermal tumors. At its limit, the degree of basal folding is such that the ratio of the area of the basal layer to the area of the overlying stratum corneum reaches about 4.5:1 (*25, 26*).

In other tissues in phase 2, the basal mitotic layer does not fold (or double) in this way. Thus, for instance, in sebaceous glands, when all the basal cells are in the mitotic cycle and phase 2 begins, the basal layer simply expands like the surface of an inflating balloon (*9*). From that point onward the area of the surface, and therefore the volume of the gland, is a function of the mitotic rate.

The conclusion is that in phase 2 the mass of a tissue, whether it is normal or hyperplastic, is determined first by the chalone concentration which fixes the mitotic rate and therefore the degree to which the basal layer folds (or doubles or expands), and second by the shorter time taken by the increased number of postmitotic cells to age and die. Evidently the relationship rate(mitosis):rate (aging) = K still holds, and this sets a firm limit to any increase in the epidermal thickness.

Regeneration

A raised mitotic rate leading to hyperplasia, whether transient or chronic, is the automatic epidermal reaction to any type of injury, whether chemical, physical, or viral. It seems probable that in most if not all cases this reaction is the outcome of damage to the cell membrane, leading to an abnormally high rate of chalone loss; the cells adjacent to an epidermal wound may lose about 50% of their normal chalone content (*13*). In addition, if the outer cell layers are destroyed, there must be an overall decrease in the chalone content of that region of the epidermis.

The consequence is that the basal cells increase their mitotic activity, which involves both an increase in the speed of the mitotic cycle and an increase in the number of mitotic cells per unit area. Some distal cells may also revert to mitosis. The epidermis may then pass out of phase 1 into phase 2 or, if it is already in phase 2, it will progress to a higher level in that phase. This response can be inhibited by treatment with epidermal chalone (*27*).

When the epidermal mitotic rate reaches a high level, the newly forming postmitotic cells have a life expectancy of only 4 or 5 days. If the damage is chronic, this sets a limit to the increased epidermal thickness. If the damage is a single episode, the decreased postmitotic lifespan can only be assumed, since the damage is repaired before the cells begin to die. In the restored epidermis the cells become normal again, their chalone content increases, their mitotic rate decreases, and their postmitotic lifespan returns to its normal length.

Clearly, the decreased postmitotic lifespan that accompanies the increased mitotic rate is a critically important safety factor which sets a tight limit to the increase in tissue mass in cases of chronic damage.

The Dichophase Ratio

It has been emphasized that an increase in epidermal mitotic activity involves not only a shortening of the mitotic cycle but also an increase in the number of cells in the mitotic cycle per unit area of skin. In phase 1 this increase in mitotic cell number is achieved by the displacement of the basal postmitotic cells; in phase 2 it is achieved by the folding (or doubling) of the basal layer. It is important to consider the way in which these extra mitotic cells are produced.

In normal epidermis it is obvious that, on average, each mitosis must produce one new mitotic cell and one cell that is postmitotic and therefore destined to age and die. It has sometimes been suggested that this ratio is automatically ensured because, after each mitosis, the two daughter cells ". . . are unequal in size, form and function, and have a different subsequent development." (*28*). There is in fact no evidence that this is so, and it must be concluded that the daughter cells emerging from the dichophase achieve the observed ratio of 1:1 for mitotic to postmitotic cells only on average.

This is called the "dichophase ratio" and its existence implies the presence of some control mechanism.

On the simple negative-feedback theory it could be postulated that a normal dichophase ratio is dependent on a normal chalone concentration. With a subnormal chalone concentration the dichophase ratio would become >1 mitotic cell to <1 postmitotic cell; with a supranormal concentration the reverse would occur. The facts, however, are more complex than this.

It is obvious that the normal 1:1 dichophase ratio must be operating in all types of steady-state epidermis, irrespective of whether conditions are normal or there is chronic hypoplasia or hyperplasia. Thus, the ratio must be stabilized irrespective of the mitotic rate and therefore irrespective of the chalone concentration.

However, whenever the chalone concentration is actually changing, the dichophase ratio must become temporarily abnormal; in no other way can the number of cells in the mitotic cycle per unit area change. For example, in normal phase-2 epidermis there may be 100 cells in the mitotic cycle in a given area of skin, while in chronic hyperplasia there may be 200 cells in the same area. These are both steady states and therefore both must show the normal dichophase ratio. The observed increase in the number of cells in the mitotic cycle can be accounted for only by the equivalent of a single division in which all the daughter cells remain mitotic.

Thus any change in the number of basal cells in the mitotic cycle is the outcome of a temporary change in the dichophase ratio which occurs in response to a changing (but not to a changed) chalone concentration.

The most obvious conclusion is that the dichophase ratio is indeed determined by the chalone concentration but that any change is rapidly and completely counteracted by some other factor as soon as the chalone concentration becomes stabilized at its new level. Nothing is yet known of any such factor, although Rytömaa and Kiviniemi (*29*) have noted the existence of an antichalone in the granulocytic system. It would be easy to construct a simple model that could account for the observed facts but at the moment this would be pure speculation.

The general conclusion is that a second function of the epidermal chalone is to promote entry into the aging pathway, and this is supported by the fact, mentioned above, that when the epidermal chalone concentration decreases to an abnormally low level the postmitotic cells of the stratum spinosum revert to the mitotic cycle.

Phase-3 Carcinomas

The upper end of phase 2 includes static epidermal tumors, such as papillomas, in which the dichophase ratio is still normal. Phase 3 is characterized by a dichophase ratio which is unable to regain normality, so that more than half the daughter cells continue to be mitotic (text-fig. 2). Frankfurt (*30*) has described carcinomas of mouse forestomach (which is epidermis-like) in which ". . . the percentage of mitotic cells that were destined to become postmitotic and mature was from 17–34% as against 50% in the normal steady state."

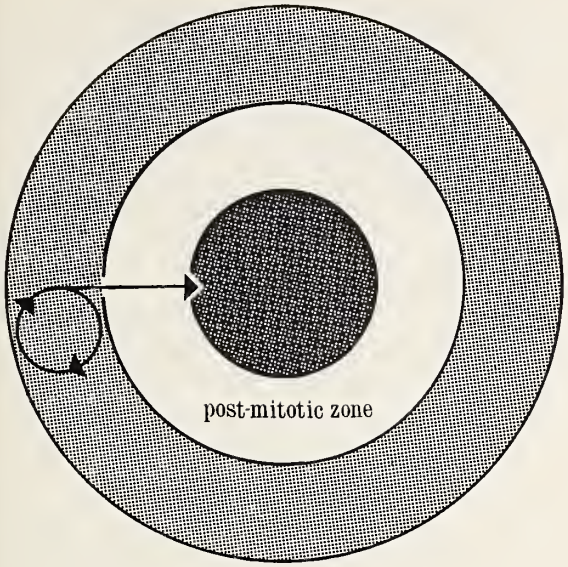
The implication is that the abnormal dichophase ratio may be the consequence of a chalone concentration which has fallen so low that the effect can no longer be counteracted as it is in phase 2.

Support for this comes from studies of the chalone mechanism in a range of actively growing tumors, including epidermal carcinomas in three different species (*8, 20, 31*). First, all tumors so far tested continue to produce the chalone of their tissue of origin and to respond normally to that chalone; second, evidence from an epidermal carcinoma and a granulocytic leukemia shows that these tumor cells have a chalone concentration $<10\%$ of normal (*cf.* about 50% adjacent to a wound); third, in 2 epidermal carcinomas and 1 granulocytic leukemia the blood content of the relevant chalone is abnormally high, due apparently to an abnormally high rate of loss from the tumor cells; and fourth, in the three cases so far tested (2 melanomas and 1 granulocytic leukemia), continued treatment with chalone-containing extracts destroyed the tumors, in some cases permanently.

Tumor growth may also be self-inhibited. When a tumor is allowed to grow unhindered, the consequence of the steadily increasing quantities of chalone that are secreted into the blood are twofold [(*20, 31*); *see* (*32, 33*)]: first, the increasing chalone concentration progressively decreases the mitotic activity of the tissue of origin, and second, as the chalone concentration increases still higher, it progressively inhibits the mitotic activity of the tumor itself. The recent work of Bichel (*33*) has shown how this type of reaction gives rise to the typical sigmoid-type tumor growth curve (*34–36*). In this connection it is important to note that, when the increasing chalone concentration induces cell death in a tumor, this death occurs normally after passage along the aging pathway (text-fig. 4) and that it may be accompanied by tissue function, as in a keratinizing epidermal carcinoma (*31*). Even if the tumor cells have lost their capacity for tissue function they still die via the aging pathway, as in a nonkeratinizing epidermal carcinoma (*37*).

In some cases, tumor growth may plateau before the animal dies and the condition then becomes chronic. Evidently, the increased chalone concentration has restored the normal dichophase ratio.

However, tumor growth is also inhibited by factors other than the chalone. Indeed, the conditions in which a tumor grows are probably always difficult because of such factors as an inadequate blood supply, chromosomal and metabolic abnormalities, and, perhaps most impor-



TEXT-FIGURE 4.—Diagram of the structure of a partly grown tumor in which the postmitotic cells show normal aging leading to death centrally (*cf.* text-fig. 1).

tant, attack by antibodies. Thus a growing tumor must be regarded as a group of cells with such a high mitotic potential (i.e., such a great distortion of the dichophase ratio) that it is able to overcome the adverse conditions. It follows that, when a tumor stops growing, this must be due to the combined effects of the adverse circumstances and the increasing chalone concentration; a tumor may therefore stop growing at a lower chalone concentration than would be needed if the conditions were ideal.

It also follows that the total destruction of a tumor by chalone treatment (*38,39*) is probably due not to chalone itself but to a composite reaction. The chalone-induced reduction in the mitotic potential may be all that is needed to allow the various adverse conditions, especially perhaps the immune reaction, to become dominant. In an essentially similar manner, penicillin prevents bacterial multiplication and so allows the normal body defenses to destroy the infection.

The destruction of a tumor in such a manner does not of course involve the coincident destruction of the tumor's tissue of origin. At the extreme, this tissue can only sink into phase 1, within which no further shrinkage is possible; with the cessation of the treatment, it immediately returns to normal.

CONCLUSIONS

The epidermal chalone delays the passage of cells around the mitotic cycle and along the aging pathway, and its action is most easily assayed in terms of inhibition of S phase or mitosis. The chalone does not inhibit the general metabolic processes.

Mitotic inhibition is so exactly matched by the inhibition of postmitotic cell aging that the ratio rate(mitosis):rate(postmitotic aging) is a constant.

In phase-1 type epidermis (e.g., mouse) the basal layer contains both mitotic and postmitotic cells; after each mitosis, a neighboring postmitotic cell is pushed out. Since the rate at which this cell ages is proportional to the mitotic rate, the epidermal thickness remains constant irrespective of the mitotic rate.

As the epidermal mitotic rate increases, the duration of the mitotic cycle decreases and the number of basal cells that are in the mitotic cycle per unit area of skin increases. Phase 1 ends when all the basal cells are in the mitotic cycle.

Any further increase in the mitotic rate, producing still more basal mitotic cells, leads to increasing lateral pressure and to the folding (or doubling) of the basal layer. Since the number of distal postmitotic cells remains in proportion to the number of basal mitotic cells, the epidermis thickens in proportion to the degree of folding. This is phase-2 epidermis (e.g., man) in which thickness is proportional to the mitotic rate.

Throughout phase 2 the average chalone concentration progressively decreases but, at each point on the scale, whether normal or hyperplastic, the epidermis is in a steady state. On average, the product of each mitosis is one mitotic and one postmitotic cell; this is the normal dichophase ratio.

When a changed mitotic rate leads to a change in the number of basal mitotic cells per unit area, this is achieved by a temporary change in

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the dichophase ratio. Thus a decrease in the chalone concentration results in more cells reentering the mitotic cycle and fewer cells entering the aging pathway. The greater the decrease in the chalone concentration, the greater the temporary distortion of the dichophase ratio.

It follows that the epidermal chalone must act to promote entry into the aging pathway.

However, as soon as the change in the chalone concentration is completed, the dichophase ratio returns to normal, which suggests the intervention of some other, still unknown, factor.

If the chalone concentration decreases to too low a value, the dichophase ratio is unable to return to normal and the result is a growing tumor. This is phase-3 type growth, which can be inhibited by chalone treatment. The tumors so far studied continue to produce the chalone of their tissue of origin and to respond normally to it; they seem to lose their chalone at too high a rate across damaged cell membranes.

There is reason to believe that these conclusions are applicable to tissues and to tumors in general.

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THE LEUKOCYTE CHALONE

Review of Leukocytes [1]

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THE TITLE, "Review of Leukocytes," is rather broadly interpreted by me to mean to try to define the parameter of the hematopoietic system with which this part of the conference will be concerned. With the hematopoietic system, we are in a mixed situation, compared with the skin epithelium, insofar as it is a much more complex population. Indeed, it is a whole chain of interlinked populations but, in recent years, a number of techniques became available which allow a quantitative analysis of the system. In other words, although many of the cells cannot be seen under the microscope, and we cannot call them by their "proper" name, nevertheless we can have perfectly good operational definitions which allow determination of absolute cell counts in the system, in organs, or in the whole body and measurements of their turnover rate using thymidine suicide and of their growth rate, population doubling time, and, in many cases, even their rate of differentiation—i.e., transit per unit time of one cell type into its descendant population.

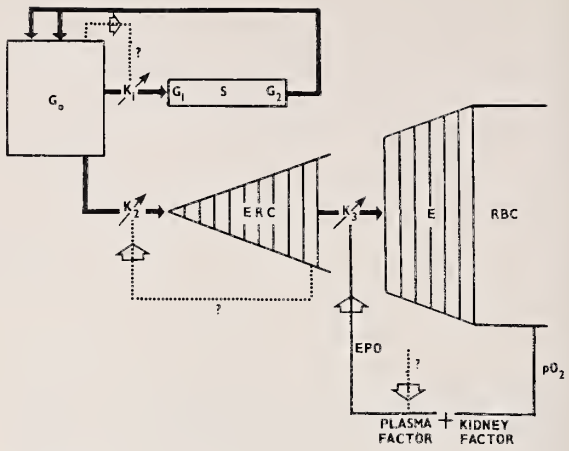
We know now that the hematopoietic system starts with a pluripotent stem cell which is more pluripotent in the mouse than in man. This pluripotent stem cell (the current abbreviation for it is CFU, or colony-forming unit) in the mouse is certainly the ancestor of all hemic cells —i.e., erythrocytes, granulocytes, platelets, and also lymphocytes (both the thymic and the bone marrow lymphocytes). In the case of man, the pluripotency is somewhat more limited because in man the lymphocyte population apparently is not coming from the same stem cells as the granulocytes, erythrocytes, and platelets. Certainly, in the small rodent, the pluripotent stem cell gives rise to some committed cell populations which people have called "secondary stem cells." The best name perhaps would be "committed transit population"; this is a population which is already committed into eventual differentiation into one of the "recognizable" cell lines (e.g., erythrocytic or granulocytic) but itself is not yet a morphologically recognizable precursor. Furthermore, at least in the erythrocyte system, this "committed" cell line requires another specific stimulus (the humoral factor, erythropoietin) before it can differentiate into the recognizable erythroid system.

In this system, the only truly self-maintaining cell population—i.e., which can maintain its number for a prolonged period of time in spite of continued loss from it—is the pluripotent stem cell or the colony-forming cell. While both the committed and the differentiating recognizable cell populations can and do divide a number of times, these divisions go *pari passu* with maturation. One might even term these a kind of amplifying transit population which undergoes a kind of suicide differentiation which as far as we know is not reversible. During these transit divisions, they amplify their number; their cell cycle time and the number of divisions during this transit can change on demand.

Text-figure 1 shows the outline of this scheme, particularly as it refers to the erythrocytic system. Here, we have the pluripotent stem cell population which is drawn as a large G_0 population, but I fully agree with Professor Bullough that the distinction between G_0 and a very long tail of long G_1 is a question of semantics. Never-

[1] Presented at the First Symposium of the International Chalone Conference, Brook Lodge, Augusta, Mich., June 5–7, 1972.

theless, a large proportion sitting in the state of "no cell cycle" (I will give the evidence for that later) is present. At any time, a small proportion can and is triggered into cell cycle due to a physiological "removal" of cells from the population. This removal is the first differentiation step which turns the pluripotent stem cell into the first committed cell population, which in this case is called the "erythropoietin-responsive cell (ERC)." In the case of the granulocytic series, the corresponding cell population may well be the agar colony-forming (or in vitro colony-forming) cell. The ERC is a committed cell population which undergoes a varying number of divisions during its transit. It has an age structure (it "matures" with successive cell cycles) and the capacity for further differentiation is very different in the "early" as opposed to the "late" ERC. These populations (the ERC certainly) have to be hit by a second stimulus for differentiation, which in the case of erythropoiesis is reasonably well characterized. This is the humoral factor, erythropoietin, which transfers ERC from the last stages of amplification into a new amplifying transit population which is then cytochemically recognizable as the erythron.



TEXT-FIGURE 1.—Cell populations involved in erythropoiesis. The pluripotential stem cell, partly in G_0 state or in cell cycle, is controlled by a local feedback control system at rate K_1. K_2 is the rate at which such cells differentiate into the committed cell population, which has its own cycle length control system as well as a signal for differentiation from the pluripotent stem cell. The nature of this is not known, but evidence indicates a mechanism wider than local. Finally, erythropoietin induces second-step differentiation at rate K_3, to produce the erythron which has both a built-in suicide mechanism of maturation as well as control of cell cycle length; the nature of the latter is not properly understood.

I have put on this scheme some arrows indicating some of the controls which must operate in this system. The solid line indicates the erythropoietin cycle. We know that the mature erythrocytes are responsible for the maintenance of appropriate oxygen tension in the tissues; with changes in P_{O_2}, a certain kidney factor is elaborated which, associated with a plasma factor, produces erythropoietin which acts on the late ERC population primarily. I said "primarily" because erythropoietin nevertheless has some detectable effect on the existing erythroid cells as well.

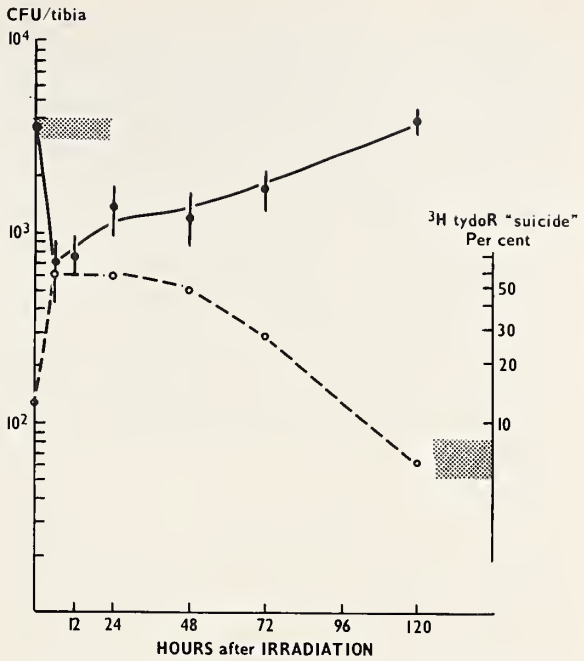
We know that the ERC population originates from the pluripotent stem cells and furthermore that there is some population-size sensor in this population, which, when the numbers decrease, increases the rate of differentiation or rate of entry of pluripotent stem cells into the committed cell population. This can be checked quite easily by causing a large-scale removal of these cells. For example, a large dose of erythropoietin "removes" ERC into the erythron. As a result, an immediate increased flow of pluripotent stem cells into the committed stem cells will follow. Consequently, of course, the CFU population will be depopulated. Its own population-size sensor will then start triggering cells into cell cycle, which of course one can pick up and demonstrate with the thymidine suicide method.

The population-size control of the pluripotent cells seems to be local in nature, as indicated by experiments in which we irradiate the whole animal, say with 600 rads, while shielding one tibia. In this experiment, about 95% of the whole hematopoietic system is being irradiated. Of course, we will kill a large proportion of the CFU and ERC as well in the irradiated areas. As a result, we would expect a general demand for increased differentiation from any existing or remaining stem cell population into the ERC. Naturally, in the irradiated areas of the

bone marrow, the number of these cells will be immediately depleted, but also, as long as the signal from the ERC population for differentiation is general, not local, in nature, we would expect a temporary depletion of pluripotent stem cells even in the shielded area. Furthermore, since we know that the proliferative capacity of the ERC population is elastic and the number of cell divisions in it can vary on demand, we can expect (and also experimentally demonstrate) that this population can increase its size to near-normal limits even when the pluripotent CFU population is still well below normal levels. As soon as the ERC population all over the body reaches near-normal size, the demand for increased differentiation will stop and therefore, in the shielded, unirradiated, 5% of the marrow, the CFU should come to a rest (i.e., a noncycling state). That this is the case is illustrated in text-figure 2 which shows that the stem cell numbers and turnover rate can be normal in a piece of shielded bone marrow even when in 95% of the hematopoietic system the numbers are low and the turnover is high.

With the thymidine suicide method, we can check at any stage the proliferation state of all these various cell populations. Normally, the CFU, as I have said, consists of a primary G_0 type of population and it is said that in a "clean" mouse (without infection) the suicide figure is rarely above 10%; it is between 5 and 10% in most cleanly kept strains. During any process of regeneration, whether it is affected by previous irradiation or by increased demand for committed cell production, the suicide figure can get up to about 50%, indicating that a large proportion of these cells can be triggered into cycle. Furthermore, this can be effected not only in vivo but also in vitro: In short-term incubation of bone marrow stem cells with cyclic AMP or isoproterenol in as low a concentration as 10^{-10} or 10^{-14}M, respectively, over 50% of the CFU will be triggered into the DNA asynthetic phase of the cell cycle within about 1 hour. Clearly, here we are dealing with a population which is exquisitely sensitive to demand for proliferation as well as for differentiation.

We also know that, when it is actually proliferating under demand, it cannot proliferate



TEXT-FIGURE 2.—Colony-forming unit (CFU) number and turnover rate in shielded tibia after 600-rads total-body irradiation. *Solid line* = number of CFU per tibia; *interrupted line* = thymidine suicide. *Note* that, in the unshielded part of the bone marrow (femur or tibia) at 120 hours after 600 rads, the CFU are still well below 10% of normal (*hatched area*) and the cycling rate is around 50%.

in an unchecked fashion. From the actual growth of splenic colonies in irradiated grafted animals, we can clearly demonstrate that even under maximal demand for proliferation there is also a differentiation—i.e., loss of cells from this population for differentiation. There appears to be an upper limit for this loss per cell cycle, as indeed one would expect. Otherwise, under maximal demand for differentiation, the grafting of bone marow into an irradiated mouse would result in a "last glorious fling" for differentiation, all the stem cells would disappear, and the mouse would not recover. The fact of course is that the mouse recovers, and these grafted bone marrow stem cells "take." Their absolute increase in number can be measured in the grafted animal, and, from the growth curves, it appears that the maximal differentiation rate is around 38–40% per cell cycle.

Now, as far as the committed stem cell populations are concerned, they constitute an interesting and somewhat less well-defined population system. In the case of the erythropoietin-responsive cell, the curious thing is that it is a continuously cycling cell population. In a normal animal, at any time, 60–70% of these cells appear to be in DNA synthesis. Furthermore, this rate is maintained even when there is no demand for erythropoiesis—e.g., in the polycythemic mouse. What these cells cycle for, what they are producing, or how they are dying, if they cannot turn into erythroblasts, nobody knows. So far, we have not been able to identify these cells morphologically and, therefore, cannot follow their fate.

In the case of granulopoiesis, it is thought that those cells which can form in vitro colonies in the agar system constitute the committed precursors for the granulocyte population. The number of agar colony-forming cells can be assayed, and their turnover rate and growth rate can be measured in vitro or in vivo. Unlike the pluripotent stem cells and the erythropoietin-responsive committed cells, they have an intermediate range of turnover. I mentioned that in normal steady state the pluripotent stem cells have only about 5–10% of their population in S period at any time; the figures are 60–70% in the case of erythropoietin-responsive cells and 40–50% in the case of the agar colony-forming cells. Clearly, their rate of turnover is significantly different from either the pluripotent stem cells or the erythropoietin-responsive cells. Like the erythropoietin-responsive cells and the pluripotent stem cells, their turnover rate is elastic. On demand, a higher proportion than 40% can be triggered into S period; however, even under maximal demands, they never reach as high a proportion of DNA-synthesizing cells as do the erythropoietin-responsive cells.

Now turning to the final populations, from the erythropoietin-responsive cells and agar colony-forming cells, a "second step" differentiation produces the last and perhaps best known and certainly recognizable population—the erythroid or granulocytic series of cells. These are also dividing transit populations with a certain number of cell cycles built into them during transit and, like the erythropoietin-responsive cells and possibly the agar colony formers, their maturation and proliferation are partially interlinked. The best understood is the erythroid cell population. Of course, it has a convenient marker, the hemoglobin synthesis, which can be labeled and therefore in this cell type the actual rate of maturation (the rate of hemoglobinization) can be assessed. The erythroid cells undergo about five divisions, during which the hemoglobin concentration is gradually built up. At a certain critical stage of hemoglobinization, the cells reach a stage which disallows any further process of proliferation. I purposefully use the word "disallows" because this explains, I think, that we do not know exactly what happens. However, when a cell is turning into a nearly mature erythrocyte, with say 27 pg of of hemoglobin in it, it is already such a "dry" cell with so little space for cytoplasmic fine structure and organelles that it is perhaps not surprising that it cannot perform the complex metabolic procedures required for proliferation. I call this a "suicide maturation" pathway.

As with the "committed" cells, the cell cycle times can be varied during transit to some extent and also the number of divisions—in both erythroid and granulocytic "final" populations. For example, in the erythroid cells the hemoglobinization acts, to some extent, as the "break" in the otherwise exponential growth, but the time when such a break should occur will depend on the rate of hemoglobinization compared with the intermitotic cycle time. If the rate of maturation is faster relative to the cell cycle time, then, of course, the "suicide" stage (cessation of proliferation) will be realized after which the cell will "skip" the last division (will be unable to undergo the last one or two divisions). This is well attested in the erythroid system. Conversely, if the rate of maturation is slower compared with the cell cycle time (slower than in normal steady state) then extra division(s) could occur during maturation, thereby, of course, increasing the amplification and output.

In a case of the lymphocytic system, we unfortunately do not possess this kind of knowledge of the constitution of cell populations. We know

that in small rodents (unlike in man) the lymphocytes come from the same pluripotent stem cells as do all the other hemic cells. What kind of amplifying populations are interwoven between the recognizable lymphocytic cell types and the pluripotent stem cells we do not know. The cardinal difference between the lymphocytes and the other "end" cells (erythrocytes, granulocytes) is that, unlike the latter, the lymphocytes can be triggered into a cell cycle with appropriate stimuli. This is well attested by the phytohemagglutinin (PHA) and similar stimulations, although the precise number of divisions such a stimulated cell can normally undergo is not yet known.

Now, to conclude and summarize: In the hematopoietic system we are dealing with a truly self-maintaining population, the pluripotent stem cell, which has its own population-size control. This is unlikely to be controlled by its mature descendants because it is producing a host of different mature descendants. The mature granulocytes could not and certainly should not inhibit the proliferation of the stem cell, since then it would also depress, of course, erythrocyte and platelet production, and vice versa. We also know from recent experiments that the feedback control in the case of the stem cells is fairly local in nature. Then, we have the committed transit populations which are amplifying (and rapidly dividing) themselves but apparently are not self-maintaining. The only control here is the control of the length of the cell cycle and the number of cell cycles that can be undergone before death or differentiation into the next, the "final" maturing populations. There is a control of the rate of the "first step of differentiation"—the rate at which pluripotent stem cells "feed into the committed" cell populations. And, finally, the last transit population, the recognizable erythroid and granulocytic cells, similarly are cell lines which are not self-maintaining. In these again, the cell cycle length can be (within narrow limits) varied, and also the number of divisions during transit. These variations, of course, are important because one extra division or one division less would double or half the output of the population. This may be controlled by more than one mechanism, possibly connected with "maturation" processes. We do know that there are certain differentiation stimuli which operate but, of these, only one, erythropoietin, has been more or less characterized. Clearly this population complex represents a host of feedback control systems in which the term "chalone" must cover a multitude of very different controlling factors, and we must be very clear in our minds (and experimental assay systems) before we can talk about *specific* control.

Some Properties of the Lymphocyte Chalone [1, 2]

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SUMMARY—We presented evidence that there exists uniquely in lymphoid tissue a noncytotoxic, cell-specific mitotic inhibitor for lymphocyte mitosis in normal cells stimulated by phytohemagglutinin (PHA) or in human leukemic lymphocytes in culture. This activity is not species specific. This chalone is not bound to the surface of the leukemic lymphocyte nearly as well as it is to the surface of the normal lymphocyte; thus, the PHA-stimulated normal lymphocyte system is much more sensitive to this lymphocyte chalone than is the NC–37 cell system. While leukemic lymphocytes apparently do contain some mitotic inhibitor and must, of necessity, release most of this into the medium, relatively little of the mitotic inhibitor can be demonstrated in the used medium from NC–37 cells in culture. This lymphocytic chalone must be a glycoprotein containing large amounts of carbohydrate and probably is located on the surface of the lymphocyte itself. Finally, we propose that the most effective technique of isolating and partially purifying lymphocyte chalone is to extract it from desiccated, defatted spleen preparation with water and then, after precipitating the bulk of the cathepsins and other proteins with 50–70% ethanol, to use molecular sieving to concentrate, collecting the fraction between 30,000 and 50,000 Daltons. The material so prepared is a considerably purified (and quite stable) lymphocyte chalone preparation suitable for use in animal studies as well as for a primary starting material for the final isolation and purification of the lymphocyte chalone.—Natl Cancer Inst Monogr 38: 117–122, 1973.

THE FIRST application of the chalone concept, as described originally by Bullough and Laurence (*1*), to lymphocytes was that reported by Moorhead et al. (*2, 3*) in terms of the transformation of normal human lymphocytes by phytohemagglutinin (PHA) and of the spontaneous transformation of leukemic lymphocytes in culture. In each case, Moorhead et al. showed that extracts of the lymph node of the pig were capable of inhibiting to a marked degree the uptake of ^{3}H-labeled thymidine by these human lymphocytes in culture. This work was confirmed in terms of leukemic lymphocytes by Garcia-Giralt et al. (*4, 5*) and in terms of PHA-stimulated lymphocytes by us (*6*).

The active ingredient from extracts of pig lymph node and spleen has been shown by us to be a thermolabile, trypsin-digestible material with a molecular weight between 30,000 and 50,000 Daltons (*6*). This activity is soluble in 70% ethanol (*4*); a similar inhibitory activity is extractable from spleen, lymph node, and thymus of pig, cow, and rat (*6, 7*).

The primary characteristics of a chalone, according to the concept of Bullough and Laurence (*1*), is that it is an endogenous mitotic inhibitor which has no species specificity and is not cytotoxic but is tissue- or cell-specific. In a recent publication (*7*), we described the lymphocyte chalone for PHA-transformed human lymphocytes in culture. This paper reports on an extension of this work in terms of NC–37, a leukemic lymphocyte of human origin, and some further properties of this lymphocyte chalone.

[1] Presented at the First Symposium of the International Chalone Conference, Brook Lodge, Augusta, Mich., June 5–7, 1972.

[2] This investigation was supported in part by Public Health Service research grant CA12743-01 from the National Cancer Institute.

METHODS

In the primary method for assay, 10^6 mononuclear leukocytes from human volunteers are incubated with PHA-p (Difco) for 66 hours in 2 ml of medium 199 containing 20% calf serum. Then 1 μCi of ^{3}H-labeled thymidine is added to the culture system and incubation is continued for 6 more hours. These cultures are then diluted, washed once with cold isotonic saline, and precipitated with 5% trichloroacetic acid (TCA); the precipitate is washed 3 times with 5% TCA and solubilized with 0.5 ml of NCS (Nuclear Chicago Solubilizer). The radioactivity of the solution is then determined in a liquid scintillation counter. The cytologic features and viability of the cultures were ascertained, before TCA precipitation, by using vital staining (trypan blue, 0.4%).

NC–37 cells were obtained from the National Cancer Institute (National Institutes of Health, Bethesda, Md.) and grown in McCoy's medium. This medium differs from medium 199 largely in containing glucose at 400 mg/100 ml rather than at 100 mg/100 ml. The assay technique was the same for these cells as for normal lymphocytes except the thymidine-^{3}H was added for only 4 hours and no PHA was used. The NC–37 cells have been well documented to be a model for human lymphoid leukemia.

Extracts of tissues were made in essentially the same way regardless of the nature or origin of the tissue. Tissue was minced with scissors and then extracted in a sonicator with 10 ml of 0.15M NaCl/g of tissue. The resulting tissue brei was allowed to stand in the cold with stirring overnight and then was centrifuged. The resulting clear supernatant was subjected to dialysis against 200 volumes of water in the cold; after recentrifugation, the clear dialyzed fluid was lyophilized. The lyophilized material was reconstituted in the appropriate medium at a concentration of 500 μg/ml (1 mg/10^6 cells/culture).

RESULTS

The effect of extracts of a wide variety of tissues from rat, calf, pig, and human on the amount of thymidine-^{3}H incorporated by PHA-stimulated human lymphocytes or NC–37 human lymphoid tumor cells in culture is shown in table 1. These results are expressed as percentage inhibition compared to controls with each tissue without the tissue extract.

TABLE 1.—*Species and tissue specificity of inhibition of thymidine-^{3}H uptake by lymphocytes in culture*

Extract from	Normal lymphocytes (PHA)		NC-37 lymphocytes	
	Percent inhibition	Cytotoxicity	Percent inhibition	Cytotoxicity
Rat:				
Liver	6	±	15	0
Spleen	88	±	41*	0
Muscle	3	0	6	0
Lymph node	92	0	55*	0
Calf:				
Thymus	90	±	40*	0
Spleen	85	+	50*	0
Lymph node	90	0	—	—
Muscle	0	0	0	0
Pig:				
Spleen	80	±	55*	0
Lymph node	95	0	60*	0
Muscle	0	0	6	0
Brain	—	—	20	0
Kidney	—	—	12	0
Human:				
WI-38 fibroblasts	0	0	6	0

*Significant inhibition ($P < 0.05$).

Regardless of the species of origin, extracts of all major lymphoid tissues significantly inhibited the uptake of DNA precursor by both PHA-stimulated normal lymphocytes and human leukemic cells. The cytotoxicity is more noticeable with the PHA-stimulated cell system than with the NC–37 cell system. This is probably because the cells are exposed to the tissue extracts for 3 days in the former system and usually only for 4 hours in the latter system. Thus another complication of the question of cytotoxicity is offered—i.e., the duration of exposure to the presumed cytotoxic agent may mask a potential inhibition of mitosis produced only by a cytotoxicity which is not grossly apparent. In general, however, the data of table 1 confirm the concept of the chalone being relevant to the control of lymphocyte mitosis—both analytical systems indicate that the mitotic inhibition was not necessarily cytotoxic, was not species-specific, and appeared to be specific with respect to tissue of origin.

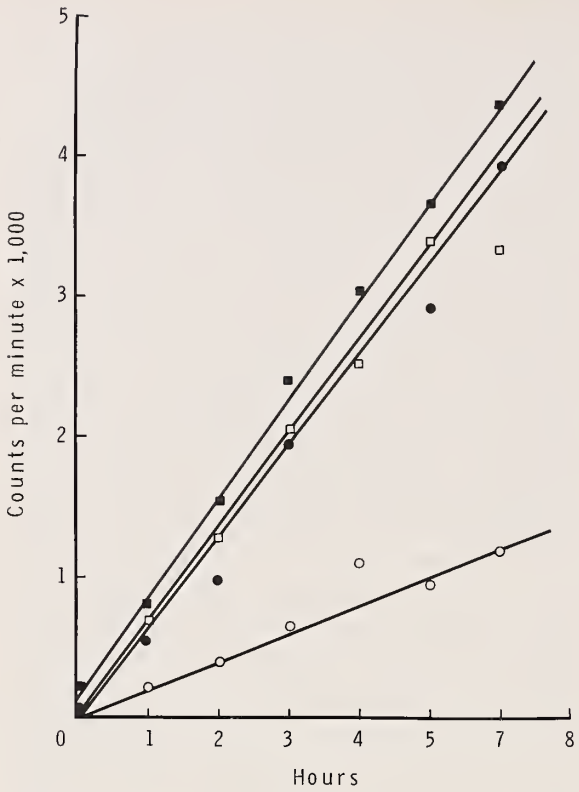
Separate experiments using lymphoid tissue extracts from both rat and cow (spleen and thymus) have indicated that the chalone extracts, once subjected to molecular filtration in an Amicon Diaflo technique as described by us previously (*6*) and hence not contaminated with cytotoxic material, had no effect on the rate of mitosis of either diploid human fibroblasts or HeLa cells in culture. This offers further confirmation of the specificity of the chalone effect at the cellular level.

We have recently found that the most reliable source of lymphocyte chalone is bovine spleen which has been extracted with 1,2-dichloroethane and dried. This preparation of defatted and dehydrated bovine spleen has an enormously long shelf-life at room temperature and contains a much higher concentration of chalone per unit weight than does fresh tissue (which is approximately 80% water and fat). This material (which is prepared for us by Mr. Ezra Levin of the Viobin Corporation, Monticello, Ill.) is extracted in distilled water at 4° C in a Waring blender and left overnight to swell; the supernatant fraction from the centrifugation is worked up in the usual fashion. We have found that this extract is considerably more active as a lymphocyte chalone source than our similar extracts prepared from fresh tissue. All of the following work was done using extracts of the defatted spleen powder prepared by Viobin.

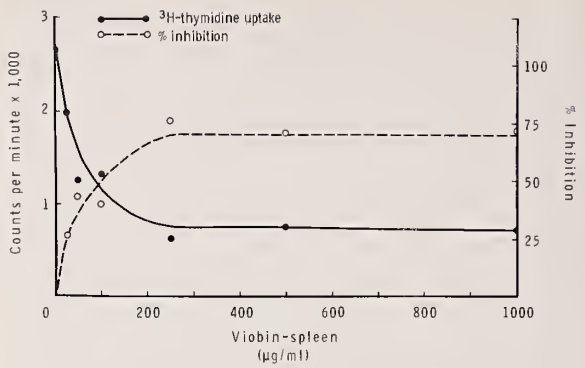
Text-figure 1 shows the kinetics of thymidine-^{3}H incorporation by NC–37 cells in the presence and absence of spleen extract (1 mg/ml), compared with the incorporation of ^{14}C-labeled phenylalanine. Even in the presence of a large amount of spleen extract, which results in a 70% inhibition of thymidine uptake at 4 hours of incubation, no significant decrease in the amount of phenylalanine uptake can be demonstrated. We have chosen to assay the NC–37 system at 4 hours, since this is the minimal time in which significant numbers of counts of thymidine-^{3}H can be demonstrated within these cells. The data of text-figure 1 indicate that, at least in terms of protein synthesis, there is no real cytotoxic component in the defatted spleen preparation. We have confirmed this observation up to 6 hours, both in terms of incorporation data and in terms of the cellular exclusion of vital dye. In general, these NC–37 cells at their healthiest are approximately 80% viable. We have found that, with a little care to collect the cells during their best growth, cultures of between 67 and 75% viability can be obtained. Experience has also indicated that use of cultures in which the viability of these cells is less than 60% can often lead to spurious results.

The effect of concentration of defatted spleen extracts on the incorporation of thymidine-^{3}H into NC–37 cells is shown in text-figure 2. The data also are recast in terms of percentage of inhibition. Beyond a certain low concentration, further increases of inhibitor concentration were without effect on the percentage of activity inhibited. This apparent saturation of the inhibitory effect of these extracts suggests strongly that any reasonable attempt to quantitate the purification of this material must be based on some parameter such as the concentration required to effect a 50% inhibition of mitotic activity (i.e., I_{50}).

Quantitatively, the defatted spleen extract will inhibit approximately 50% of the thymidine-^{3}H uptake of NC–37 cells within 4 hours of incubation at a concentration of approximately



TEXT-FIGURE 1.—Kinetics of incorporation of ^{14}C-labeled phenylalanine (*squares*) and of ^{3}H-labeled thymidine (*circles*) into NC–37 cells in presence (*open symbols*) and absence (*solid symbols*) of Viobin spleen extract (500 μg/ml).



TEXT-FIGURE 2.—Effect of concentration of Viobin spleen extract on incorporation of ^{3}H-labeled thymidine into NC–37 cells in 4 hours' incubation at 37° C.

100 μg/ml; similar saline extracts made of fresh spleen will produce a 50% inhibition of "mitotic activity" at a concentration of approximately 500 μg/ml.

Another important characteristic of the lymphocyte chalone is its binding to the cell. Table 2 compares the effect of rinsing both normal diploid lymphocytes and NC–37 leukemic lymphocytes with serum-free medium after exposing these cells overnight (18 hr) in culture to a high concentration of spleen lymphocyte chalone. In normal leukocytes the inhibitory activity of this spleen-derived chalone was not easily reversible by rinsing prior to exposure to PHA. However, the NC–37 cells seemed to lack the ability to bind the chalone inhibitor because simple rinsing completely removed any evidence of prior exposure to the inhibitor. While this finding suggests that there can be no significant cytotoxic damage to the cells after 18 hours of incubation, because mitotic activity can be completely recovered by a simple rinsing procedure, it does indicate some considerable problems in the pharmacology of using lymphocyte chalone to control leukemia in vivo. The concentration of chalone must be large to control the mitosis of these cells which lack the ability to bind the chalone to their surface, particularly when compared with the considerably smaller amounts of chalone which should be effective in inhibiting the transformation of normal lymphocytes which have been stimulated antigenically.

Preliminary data, which will be discussed by Chung later in this conference, indicate that concentrations of lymphocyte chalone which seem to be effective in delaying the allograft rejection phenomenon in rodents were without effect in controlling rat leukemic tumor growth in vivo.

The question of the ability of leukemic lymphocytes to generate chalone as well as to respond by mitotic inhibition to its presence was studied in a series of experiments. The results are summarized in table 3. We compared the ability of extracts of NC–37 cells (prepared by sonication in isotonic saline as described above) and the nondialyzable lyophilized portions of used medium from NC–37 cells in terms of their ability to inhibit thymidine-^{3}H uptake by PHA-stimulated normal lymphocytes and NC–37 leu-

TABLE 2.—*Effect on thymidine-^{3}H uptake of rinsing lymphocytes with inhibitor-free medium after incubation with inhibitor*

Condition	Uptake (cpm/10^6 cells)	Inhibition (%)	Cells viable (%)
NC-37 cells:			
Control	4,353	—	71
Spleen	1,265	71	67
Spleen (rinsed)	4,399	0	69
Normal lymphocytes (PHA):			
Control	68,000	—	95
Spleen	6,800	90	>90
Spleen (rinsed)	7,900	88	>90

TABLE 3.—*Inhibitory effects of NC-37 cell extract and of used culture medium on thymidine-^{3}H uptake by PHA-stimulated and leukemic human lymphocytes*

Extract (μg/ml)	Inhibition (%)	
	Normal lymphocytes (PHA)	NC-37 lymphocytes
NC-37 cells:		
1,000	74*	40*
500	42*	21
250	20	0
NC-37 medium:		
1,000	53*	19
500	23	13
250	20	13

*Significant inhibition ($P < 0.05$).

kemic lymphocytes. These results indicate that PHA-transformed systems are more sensitive to the addition of mitotic inhibitor than are NC–37 cell systems, a finding consistent with the data indicating that NC–37 failed to bind the lymphocyte chalone effectively in culture. Also, it is apparent that there is a significant amount of inhibition of lymphocyte transformation, in either normal or leukemic lymphocytes in culture, by extracts of NC–37. The amount of inhibitor that can be demonstrated in the NC–37 cell extract is considerably larger than the amount of inhibition that can be demonstrated by the used medium derived from these cells. Since it is known that NC–37 cells would not bind the chalone and presumably would release it all into the medium, this is a surprising finding. It is not clear whether it reflects that very little chalone is being made by NC–37 cells or that the chalone is destroyed, once it is released into the medium, by proteases which have also been released from these cells during the culture period. Our impression is that the NC–37 cells make relatively little chalone when compared with the amount of lymphocyte inhibitor that can be demonstrated to be made by normal cells. We conclude then that an important difference between the cancer cell and the normal lymphocyte is that the leukemic cell does not make as much chalone and will not bind chalone as effectively as does the normal cell.

We have investigated some of the general properties of lymphocyte chalone. We have shown that its molecular weight must lie between 30,000 and 50,000 Daltons by the Amicon-Diaflo molecular sieving technique (*6*). LaSalvia et al. (*5*) have shown that the activity is soluble in 70% ethanol. We have confirmed that all of the mitotic inhibitory activity that can be extracted from spleen and thymus is soluble in 70% ethanol. We have also found that a significant but small amount of activity can be recovered by extracting lymphoid tissues in the cold with either 5% TCA or 1M perchloric acid! The amount of activity that is recovered by the extraction of spleen or thymus with these rigorous solvents is relatively small, but the surprising fact is that any activity at all can be recovered.

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In the case of the TCA extraction, the extract was neutralized to convert the TCA to chloroform and CO_2 and dialyzed exhaustively against water in the cold. In the case of the perchloric acid extraction, the extract was merely dialyzed exhaustively against 200 volumes of water in the cold. After this dialysis, the supernatants were lyophilized and assayed. Their specific activity was not larger than that of the saline extracts from which they were prepared, but the finding that significant amounts of this activity were soluble, despite being denatured in large part in such solvents as perchloric acid and TCA, indicates very strongly that the chemical nature of lymphocyte chalone must be a glycoprotein. Some lipoproteins might also be soluble in these solvents but, without exception, their molecular sizes are vastly in excess of 50,000 Daltons. Thus it would appear that the lymphocyte chalone is, in fact, a glycoprotein containing very considerable amounts of carbohydrate.

Finally, we have devised what we think is an extremely useful technique for the isolation and partial purification of the lymphocyte chalone. Extracts of the defatted, dehydrated, bovine spleen are prepared, with 5–10 ml of distilled water/g of spleen powder, with a Waring blender in the cold for 2–5 minutes. Then the suspension is allowed to stand in the cold with stirring for 18 hours (overnight). This latter step is essential to permit fully effective extraction of lymphocyte chalone. It makes no difference whether the defatted spleen powder is extracted in isotonic saline or in water and whether this is done in a Waring blender or by sonication.

After it has stood overnight in the cold, the mixture is centrifuged in the cold and the clear supernatant is removed. Since the fat has been removed by the 1,2-dichloroethane extraction technique used to prepare the powder, there is no problem about lipids floating on the surface of these aqueous supernatants. The centrifuged material is placed in an Amicon-Diaflo sieving device and expressed through a 50,000 Dalton filter. Material which passes through this filter is then concentrated by using a 30,000 Dalton filter. This clarified supernatant is then mixed with at least an equal volume of ethanol in the cold. The resulting mixture is allowed to stand for an hour and then is centrifuged; the clear supernatant at approximately 50% ethanol concentration is removed and dialyzed against 200 volumes of water in the cold for 24 hours (long enough for most of the alcohol to be removed).

The final aqueous material, with a molecular weight in the range of 30,000–50,000 Daltons, is lyophilized. The final product is purified some 80 to 100 times compared to the starting material. This product, which is not pure or homogeneous, containing as it does a variety of glycoproteins in addition to the lymphocyte chalone, is nonetheless completely free of cytotoxic materials. Relatively small amounts of this material have significant inhibitory effects on the mitosis of lymphocytes in culture—e.g., 20 μg/ml gives a 50% inhibition of PHA transformation in normal lymphocytes.

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Commentary on "Some Properties of the Lymphocyte Chalone" by J. C. Houck [1]

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THIS WORK by Houck and his colleagues has several remarkable aspects. Perhaps one of the most important is that it confirms the existence of a specific DNA-synthesis inhibitor from lymphocytes. In the paper which follows, I will give some of the data presently known about our lymphoid extract. In regard to its molecular weight, its in vitro activity as an inhibitor of DNA synthesis, its lack of systemic toxicity, its heat lability, its trypsin sensitivity, and its lack of detectable effect on organs other than lymphoid, our results are totally in agreement with those of Houck. However, I would like to stress one difference which I think is fundamental to the theoretical consideration of the chalones.

Since the start of our work we have been interested in the problem of tissue specificity, and at first we attempted to find that specificity in vitro. Certainly, the extracts of both bovine spleen and human lymphocytes had inhibitory activity. But, we found that our aqueous spleen extract could inhibit the proliferation of permanent cell cultures of diverse origin. After alcoholic fractionation, the extract exhibited at least two bands on polyacrylamide gel electrophoresis and showed no more specificity.

In collaboration with Dr. Bizzini of the Pasteur Institute in Paris, who further purified the alcoholic extract by preparative electrophoresis, we were able to demonstrate the inhibitory activity to be tenfold more active in one homogeneous band. Unfortunately, we have not been able to obtain enough purified product to test its specificity against a panel of a large number of diverse tissues.

Several investigators, including Iversen and Rubin, have raised the question of whether the inhibition seen in tissue culture is comparable to the alleged in vivo mechanism of regulation. Recently, the work of Prunieras's group in Paris, who are interested in the epidermal chalone, showed that specificity in vitro can be modified by the enzyme treatment necessary to establish a primary cell culture.

We have evidence demonstrating in vivo tissue specificity of the alcohol extract. Animals given injections of the product show a decreased uptake of tritiated thymidine in peripheral lymphoid tissue, but there is no effect on nonlymphoid organs such as liver and kidney. Furthermore, bone marrow cells obtained from animals that had received injections show no apparent inhibition in hematopoietic ability as measured by spleen colony formation in irradiated recipients, while spleen and lymph node cells show a decreased proliferative ability, as measured by PHA and MLC stimulation and, in vivo, by GVH and transfer procedures.

[1] Presented at the First Symposium of the International Chalone Conference, Brook Lodge, Augusta, Mich., June 5–7, 1972.

Extraction From Bovine Spleen of Immunosuppressant With No Activity on Hematopoietic Spleen Colony Formation[1]

Emilio Garcia-Giralt, W. Rella, V. H. Morales, E. Diaz-Rubio, *and* F. Richaud, *Institut de Cancérologie et d'Immunogénétique, Hôpital Paul Brousse, 94800-Villejuif, France*

SUMMARY—An alcohol extract of calf spleen inhibits lymphocyte proliferation and has the in vivo characteristics of a lymphoid chalone. It prevents the graft-versus-host reaction when given to the parental cell donors or controls it when given to the F_1 recipients. Its effect on hematopoietic spleen colonies was studied. It seems that the development of colonies in X-irradiated mice given injections of bone marrow cells could not be altered by administration of this substance.—Natl Cancer Inst Monogr 38: 125–129, 1973.

RECENT DATA suggest that the rate of proliferation, in most tissues studied thus far, is controlled by tissue-specific inhibitors of mitosis and DNA synthesis (*1–5*). These chalones are active both in vivo and in vitro, and their action appears to be reversible. Similarly, the circulating lymphocyte may eventually produce its own specific inhibitor which, passing through the blood, would suppress the lymphocyte production rate.

In 1969, Moorhead et al. (*6*) presented evidence that aqueous extracts of pig lymph nodes can inhibit the in vitro transformation of human lymphocytes induced by phytohemagglutinin (PHA). In 1970, we reported (*7*) the inhibitory effect, in the same alcoholic fraction, of aqueous extracts of human lymphocytes and calf spleen cells on DNA synthesis in established cell lines from the peripheral blood of normal donors. RNA and protein synthesis were not inhibited. The active component appeared to be a protein with a molecular weight of 45,000. The alcoholic fraction of bovine spleen (S4) was used in all subsequent experiments. We reported the inhibition of tritiated thymidine incorporation into transformed human lymphocytes stimulated by PHA or by histocompatibility antigens in the mixed lymphocyte culture. In vivo, we found that S4 was able to inhibit cell proliferation of murine lymphoid tissue specifically and to decrease the number of immune competent cells in the spleens of mice immunized with sheep red blood cells (*8*).

Because of its apparent ability to prevent cell-mediated immunity in vitro, we studied the effect of S4 on the graft-versus-host reaction (GVH) (*9*). S4 was shown to prevent (when administered to the donors) or to control (when given to the recipients) the acute GVH induced in sublethally X-irradiated (DBA/2 × C57Bl/6)F_1 mice transplanted with bone marrow (BM) and lymph node (LN) cells from C57Bl/6 mice. S4 did not display systemic toxicity or intolerance when given intravenously, intramuscularly, or intraperitoneally. Most of these results have been confirmed with lymphoid extracts of different origins (*10–12*) and concern the proliferation of stimulated lymphocytes or lymphocytes involved in immune processes.

[1] Presented at the First Symposium of the International Chalone Conference, Brook Lodge, Augusta, Mich., June 5–7, 1972.

We now report data on the action of S4 on the hematopoietic stem cell compartment (defined as cells capable of forming colonies in the spleens of lethally irradiated mice) and on its effect on the growth of these hematopoietic colonies. The results reveal that, while the lymphocytes of S4-treated mice are not capable of blastic transformation when stimulated by PHA, are not capable of restoring the hemolytic antibody response to sheep erythrocytes in immunosuppressed recipients, and are not capable of producing an acute GVH in the irradiated host, S4 treatment of mice does not alter the ability of their hematopoietic stem cells to form colonies in sublethally irradiated recipients.

MATERIALS AND METHODS

S4 preparation.—Calf spleen, obtained from animals 1–2 years old, was dissected and the capsule and fibrous tracts were removed. The cells from the remaining tissue were washed 3 times with saline, resuspended in 100 ml of distilled water, and left for 1 hour. The suspension was then homogenized for 1 minute with an Ultra-Turrax homogenizer, left 30 minutes, and then centrifuged at 17,000 × *g* for 45 minutes. The pellet was discarded and the supernatant was dialyzed against deionized water for 24 hours. The precipitate resulting from dialysis was eliminated by centrifugation. Ethanol (96%) was then added dropwise to the supernatant until a 75% ethanol concentration was reached. The suspension was centrifuged at 10,000 × *g* for 20 minutes; the precipitate was discarded and the supernatant was evaporated under vacuum. This fraction containing 2–10 mg of protein/ml was designated as "S4." All the extraction procedures were performed at 4° C. Heat-inactivated S4 (1 hr at 65° C), designated as "HIS4," was used as control.

Transformation by PHA.—Spleen cells from 8-week-old C57BL/6 mice were passed through a nylon mesh column and counted in a hemacytometer. The lymphocytes were suspended in 2 ml of medium at a concentration of 5×10^6/ml in 16- by 125-mm Falcon plastic screw-top tubes and incubated at 37° C in humidified 5% CO_2 atmosphere for 72 hours. The nutrient medium was RPMI supplemented with 5% heat-inactivated AB human serum. PHA-P (Difco) was added at the beginning of the incubation, to a final concentration of 10μg/ml. Tritiated thymidine (1μCi/ml) was added for 24 hours on the 2d day of culture. The cultures were centrifuged separately, washed 3 times with perchloric acid (PCA) (2%) at 4° C, resuspended in 3 ml of 15% PCA, and incubated at 65° C for 4 hours. The material obtained was then centrifuged, and 0.1 ml of the supernatant was added to 10 ml of scintillation liquid. Radioactivity was measured in a Packard liquid scintillation spectrometer.

Restoration of hemolytic antibody response to sheep erythrocytes in immunosuppressed mice.—C57BL/6 adult mice weighing approximately 20 g were given intraperitoneal injections of S4, 6 mg per animal per day from day 4 to day 1 before sacrifice. Lymphoid cells derived from the spleens of treated as well as non-treated mice were transferred intravenously to syngeneic recipients treated 24 hours earlier with 850 rads of total-body irradiation.[2] The mice were then challenged by intraperitoneal inoculation of 4×10^8 sheep erythrocytes. The animals were killed 9 days later and the numbers of antibody plaque-forming cells (PFC) in the spleens of these recipients were determined by the direct hemolytic plaque assay in agar gel (*13, 14*). The number of zones of hemolysis on two plates for each spleen cell suspension was enumerated and used to calculate the average number of PFC's per recipient group.

Graft-versus-host reaction (GVH).—A total of 30 (DBA/2 × C57BL/6)F_1 mice were exposed to 500 rads of total-body irradiation [2]; 24 hours later they were given 10^7 C57BL/6 bone marrow (BM) cells and were randomized into 3 groups of 10 each. Group A received 2.5×10^7 normal C57BL/6 lymph node (LN) cells intravenously. Group B was given 2.5×10^7 C57BL/6 LN cells from mice treated daily with 6-mg intraperitoneal injections of S4 from day 4 to day 1 before sacrifice. Group C received 2.5×10^7 LN cells from mice treated daily with intraperitoneal in-

[2] Total-body X-irradiation; 200 kV; 12 mA, TSD, 50 cm; 0.5 mm Al + 0.5 mm Cu filtration.

jection of 6 mg of HIS4 from day 4 to day 1 before sacrifice.

Colony-forming hematopoietic stem cells (CFU).—The colony-forming test was performed by intravenous injections of femoral BM cells into heavily irradiated[2] (850 rads) C57BL/6 mice; the spleen colonies were counted on the 9th day. BM suspension was made by flushing the femurs of the donor animals with Hanks' solution. Appropriate dilutions were prepared in Hanks' solution so that 5×10^4 BM cells were injected in a volume of 0.4 ml.

RESULTS

Previous studies (*15*) had shown that lymphocytes of animals treated by several injections of S4 incorporate less tritiated thymidine when stimulated in vitro by PHA or by allogeneic lymphocytes in the mixed lymphocyte culture.

In an experiment performed with lymphocytes from mice treated with S4 (6 mg/mouse) during 4 consecutive days before sacrifice, there was a decrease of 55% in their capacity to undergo transformation by PHA (table 1). Spleen cells of the same S4-treated animals were not able to restore the hemolytic antibody response to sheep erythrocytes in immune-suppressed recipients (table 2). These results can be compared with those already obtained in animals treated after inmmunization with sheep erythrocytes (*8*). In that case, the number of hemolytic plaques had been decreased by 50%.

Text-figure 1 gives the data obtained when spleen cells of S4–treated animals were used to produce acute GVH response in irradiated mice. The curves show that 30-day survival was significantly better ($P<0.001$) in F_1 mice given injections of lymphocytes from S4-treated donors than in those receiving lymphocytes from untreated donors; thus S4 decreases the capacity of parental cells to induce a GVH in the F_1, while lymphocytes from HIS4-treated animals were still able to produce it.

When BM cells from the treated mice were used to produce spleen colonies in lethally irradiated mice, the number of colonies obtained 9 days after administration was 12.4 ± 2.5, which is not statistically different from the number of colonies obtained with BM cells from untreated animals (13 ± 2.1).

Furthermore, in lethally irradiated recipients given injections of normal BM cells and treated with 6 mg of S4 per day from day 5 to day 9 after irradiation, the number of colonies detected in their spleens was 14.4 ± 4.2. These data are not statistically different from those obtained with the control mice given saline injections for the same period (13.2 ± 3.2).

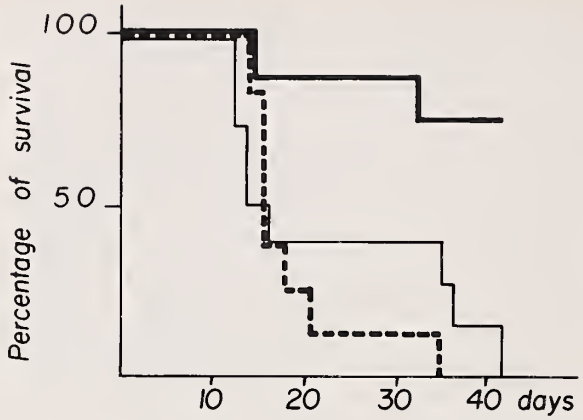
S4 does not seem to modify the engraftment

TABLE 1.—*Effect of S4 on PHA-induced transformation*

Lymphocyte source	Mean ± 95% confidence limit (N=5)	
	PHA Absent	PHA Present
Normal mouse spleen	5,269±962	44,271±4,985
S4-treated mouse spleen	4,280±286	20,967±3,190

TABLE 2.—*Effect of transfer of spleen cells from normal or S4-treated donors on antibody plaque-forming response of X-irradiated recipients immunized with sheep erythrocytes*

Cells transferred	Recipient PFC response on day 9
None	0
Spleen from normal mice	284
Spleen from S4-treated mice	56
PFC, unirradiated normal mice	679



TEXT-FIGURE 1.—Survival of 30 (DBA/2 × C57BL/6) F_1 mice exposed to 500 rads total-body irradiation and receiving, 24 hours later, normal bone marrow and lymph node cells from normal, S4, or HIS4-treated C57BL/6 mice.

A– – – : Mice receiving 10^7 BM cells from normal C57BL/6 mice and 2.5×10^7 LN cells from C57BL/6 untreated mice (saline).

B——— : Mice receiving 10^7 BM cells from normal C57BL/6 mice and 2.5×10^7 LN cells from C57BL/6 mice treated daily with intraperitoneal injections of 6 mg of S4 from day 4 to day 1.

C —— : Mice receiving 10^7 BM cells from normal C57BL/6 mice and 2.5×10^7 LN cells from C57BL/6 mice treated daily with intraperitoneal injections of 6 mg of HIS4 from day 4 to day 1. Ten mice per group.

capacity of bone marrow stem cells, as shown by the transfer of bone marrow cells of treated mice. Furthermore, as shown from the data obtained in recipient treated mice, S4 does not appear to affect the growth of the colonies. However, as far as the action of S4 on modifying the production of stem cells committed to producing lymphocytes (lymphoid stem cells ?) is concerned, no answer can be given from present data.

DISCUSSION

According to Till and McCulloch *(16)*, the term "colony forming unit" (CFU) refers to cells capable of forming macroscopically visible colonies of differentiating erythroid, granulocytic, or megakaryocytic cells in the spleens of lethally irradiated mice. These represent, very probably, only a fraction of the potential colony forming cells present in the inoculum. CFU can generate clones containing granulocytes and megakaryocytes as well as lymphocyte precursors and additional CFU.

Among the main characteristics which define a chalone *(17)*, we think that tissue specificity is the most difficult to demonstrate conclusively. Concerning the lymphoid tissue, the question of specificity is all the more complex because cells are normally present at different stages of differentiation.

The data show that the lymphocytes from S4-treated mice incorporated less tritiated thymidine when cultivated with PHA, did not restore the hemolytic antibody response to sheep erythrocytes in lethally irradiated mice, and, when injected intravenously, did not cause an acute GVH reaction in the semi-allogeneic recipient. But, on the other hand, the hematopoietic stem cells from the bone marrow of S4-treated animals did not lose their capacity to form colonies of hematopoietic foci in the spleens of irradiated mice. Furthermore, the proliferation of cells in the colonies was probably not modified when the recipients were treated with S4 from day 5 to day 9 after irradiation, since no difference in size could be detected between colonies found in treated and in untreated recipients. These findings point to a functional difference between the lymphoid and the hematopoietic stem cells, because S4, which inhibits lymphoid cell proliferation, does not affect the multiplication of erythroid, myelocytic, or megakaryocytic cells present in the colonies. Therefore, the specificity of S4 seems to be restricted to lymphoid cells.

As far as the lymphoid chalone is concerned, we found it of most interest to determine its functional specificity and to find out at what level of hematopoiesis it was acting. If, as suggested by Metcalf and Moore *(18)*, hematopoiesis is regulated by a hierarchy of chemical messages —the first one at the intracellular level and produced by regulator genes; the second at the extracellular level and produced by microenviromental agents—operating sequentially in the development and differentiation to limit the genetic potentialities of cells and if chalones are regulating the mitotic activity, thus allowing the system to adapt to environmental change, the

lymphoid chalone could be the regulator of lymphocytes stimulated or involved in immune processes.

This opens a new avenue of investigation for therapeutic utilization of this humoral regulator.

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Some In Vivo Effects of Chalone (Mitotic Inhibitor) Obtained From Lymphoid Tissues [1, 2]

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SUMMARY—Our interest in chalone was stimulated by its potential effect in mediating immune response, an effect which may have clinical application to transplantation. Our experimental data, some of which are preliminary, indicate that, when given intraperitoneally to neonatal rabbits, aqueous extracts of lymphoid tissue inhibit the rate of thymus growth in these animals. When given to the recipients (mice), these extracts prolong xenograft survival.—Natl Cancer Inst Monogr 38: 131–134, 1973.

SINCE THE reports by Bullough and Laurence on the mitotic inhibitor present in the aqueous extracts of epidermis, demonstrating that the endogenous inhibitor was heat labile, noncytotoxic, cell specific, and species nonspecific (*1–3*), subsequent investigations in several laboratories have demonstrated that specific endogenous mitotic inhibitors were obtainable from melanocytes (*4*), granulocytes (*5*), kidney cells (*6*), and cells of lens (*7*). Moorhead et al. (*8*) have shown that aqueous extracts of pig lymph nodes can inhibit the DNA synthesis and mitosis induced in vitro by phytohemagglutinin (PHA) in human lymphocytes. Lasalvia et al. (*9*) reported the inhibitory effect of aqueous extracts of human lymphocytes and calf spleen cells on DNA synthesis in human lymphocyte transformation; RNA and protein syntheses were not inhibited. Houck et al. (*10*) demonstrated that aqueous extracts of rat spleen, lymph nodes, and calf thymus and spleen inhibited DNA synthesis induced by PHA whereas the incorporation of RNA was not affected.

[1] Presented at the First Symposium of the International Chalone Conference, Brook Lodge, Augusta, Mich., June 5–7, 1972.

[2] The study is supported in part by a grant from John A. Hartford Foundation awarded to Dept. of Surgery, Georgetown University Medical School.

MATERIALS AND METHODS

Aqueous extracts were prepared from dog lymph nodes and spleen, calf thymus, pig lymph nodes, and acetone powder of rabbit thymus. These crude extracts were prepared at 4° C in 5 ml of distilled water per gram of tissue in an Omnimixer. The mixture was diluted to 1–1.5 times the volume with distilled water and centrifuged at 10,000 × *g* for 30 minutes. The fat that floated to the top was removed, and the contents of the tubes were homogenized in a glass homogenizer. The mixture was allowed to stand overnight in the cold. After the homogenate was centrifuged at 20,000 × *g* for 1 hour, the supernatant was removed and dialyzed extensively against distilled water. The dialyzed material was centrifuged again and the clear supernatant was lyophilized. These lyophilized extracts are termed "crude extracts of tissue." In the case of acetone powder, 25 ml of distilled water was used per gram of powder.

The in vitro inhibition and cytotoxicity assays on these crude extracts and partially purified fractions were performed in the laboratories of Dr. John C. Houck by their routine assay method (*10*). Some of these crude extracts were partially purified by molecular sieving through

Amicon Diaflow filters in an Amicon Diaflow chamber. The fraction retained between 30,000 and 50,000 Daltons showed a substantial purification over the crude extracts. The in vitro assay showed a 20-fold purification: Crude extracts from pig lymph nodes gave 69% inhibition at 500 μg/ml while the partially purified fraction gave 71% inhibition at 25 μg/ml.

Animals used in the studies were New Zealand white rabbits and C57BL/6 Mai mice. For the study of the inhibition, by chalone, of thymus growth, neonatal rabbits (24–36 hr) were given daily intraperitoneal injections of chalone for 5 days. Two of these animals received 3.5 mg of crude extract of acetone powder of rabbit thymus daily in 0.25 ml of saline, two received 350 μg of partially purified extract of acetone powder of rabbit thymus (30,000–50,000 Daltons), and an additional group of seven animals were given 300 μg of a partially purified pig lymph node extract (30,000–50,000 Daltons). Control animals of comparable weight were given 0.25 ml of saline daily for 5 days. All animals were killed on the 6th day. Body weights and thymus weights of the experimental group were compared with those of the control group.

Mice were chosen as recipients for xenograft survival study mainly because of size, to minimize the amount of chalone needed. Mice were given 0.25 ml of crude extracts of lymphoid tissues intraperitoneally each day; controls were given the same amount of saline. The donor skin was taken from rabbits.

RESULTS

The inhibitory effect on thymus growth in neonatal rabbits receiving chalones is shown in table 1. The body weights of the control and the experimental group were similar, but the differences in thymus weights were statistically significant ($P<0.001$).

The effect of chalone on xenograft survival is shown in table 2. The intraperitoneal injection given daily to the recipients significantly prolonged the xenograft survival time ($P<0.0001$). The result on xenograft survival needs to be confirmed with partially purified fraction (30,000–50,000 Daltons) because the crude extracts are not entirely free of cytotoxicity.

TABLE 1.—*Inhibition of thymus growth in neonatal rabbits by chalones*

Treatment	Experimental group			Control group		
		Body weight (g)	Thymus weight (mg)		Body weight (g)	Thuyms weight (mg)
Crude extract of rabbit thymus, 3.5 mg/animal/day	A	195	365	1	140	420
	B	211	216	2	176	520
Partially purified rabbit thymus extract (30,000–50,000 Daltons), 350 μg/animal/day	C	169	223	3	175	426
	D	181	283	4	222	491
Partially purified extract from pig lymph node (30,000–50,000 Daltons), 300 μg/animal/day	E	155	241	5	191	529
	F	183	328	6	175	398
	G	145	221	7	222	405
	H	195	368	8	184	381
	I	145	242	9	128	255
	J	126	160	10	125	190
	K	132	154	11	135	374
				12	185	502
Mean ± SD		167.0 ± 28.2	254.6 ± 73.7*		171.5 ± 33.4	407.6 ± 102.8

*Significantly different from control ($P<0.001$).

TABLE 2.—*Effect of crude extract of lymphoid tissue on survival of skin grafts from rabbit to mouse*

Mouse*	Days after grafting																	
	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Treated group, mean survival 12.1±3.5 (SD) days†																		
1	OK	OK	OK	OK	—	OK	OK	dead										
2	OK	OK	OK	OK	—	OK	OK	Rej.										
3	OK	OK	OK	OK	OK	OK	OK	Rej.										
4	OK	OK	OK	OK	OK	OK	OK	Rej.										
5	OK	OK	OK	OK	OK	OK	Rej.											
6	OK	OK	OK	OK	OK	OK	OK	OK	Rej.									
7	OK	OK	OK	OK	Rej.													
8	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	Rej.
9	OK	OK	OK	OK	OK	OK	Rej.											
10	OK	OK	OK	OK	OK	OK	Rej.											
11	OK	OK	OK	OK	OK	OK	—	OK	OK	OK	OK	Rej.						
12	OK	OK	OK	OK	OK	OK	—	OK	OK	OK	Rej.							
Control group, mean survival 6.2±1.2 (SD) days																		
1	OK	Rej.																
2	OK	Rej.																
3	OK	Rej.																
4	OK	OK	OK	OK	Rej.													
5	OK	OK	OK	Rej.														
6	OK	OK	OK	OK	Rej.													
7	OK	OK	Rej.															
8	OK	OK	Rej.															
9	OK	OK	Rej.															

*Mice 1 through 9 received extract of calf thymus, starting on day of grafting; mice 10, 11, and 12 received extract of dog spleen, starting 2 days before grafting.

†Statistically significant as compared to control ($P < 0.001$).

DISCUSSION

It is well known that the immune response is accompanied by active proliferation of lymphoid cells (*11, 12*). If the lymphocytes of the graft recipient were to be effectively inhibited from transforming, the immune response against the graft antigen would be severely hindered and survival of the graft may be prolonged. Data from our in vivo studies indicate that chalone can effectively suppress immune response as evidenced by xenograft survival being significantly prolonged and thymus growth of neonatal rabbits being inhibited. Garcia-Giralt et al. *(13)* reported that chalone was able to decrease the number of immune competent cells in the spleens of mice immunized with sheep red blood cells. Further evidence presented at this conference on the effectiveness of chalone in preventing the graft-versus-host reaction leaves little doubt that lymphoid chalone will play an important role in the field of tissue and organ transplantation.

However, the crucial information as to whether these mice which survived the graft-versus-host reaction (due to chalone-treated cells) were able to respond later to the challenge of sheep red blood cells has not yet been obtained. It may be speculated that one way the lymphoid chalone can be used is to give lymphoid chalone shortly before massive antigen is introduced (it could be in the form of a kidney homograft) and continue to give chalone for some time. If Burnett's clonal selection theory is correct, then the antigen stimulates the responsive clone of cells to divide; however, not all the cells of the clone are stimulated at the same time. Thus, chalone will suppress these excited cells from proliferation and hold them in phase. When all or most of these cells of the same clone are held in phase, then it is time to stop chalone injection and give an immunosuppressant, such as 6-mercaptopurine, in its place. The removal of chalone will permit these cells to proliferate at the same time and these proliferating cells will be effectively eliminated by a few large doses of 6-mercaptopurine. As repopulation of the lymphoid system

takes place, the new lymphoid cells mature in the presence of antigen and could be expected, by conventional tolerance theory *(14)*, to be tolerant. The result could be a stable state of tolerance.

The antigenic properties of lymphoid chalone have not been studied, partly because of the lack of a purified compound. It may offer some advantages over the use of antilymphocyte γ-globulin. It appears that there are numerous possibilities which need to be investigated for the therapeutic use of lymphoid chalone.

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A Lymphocyte-Inhibiting Factor (Chalone?) Extracted From Thymus: Immunosuppressive Effects[1, 2]

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SUMMARY—The partial purification of a lymphocyte-inhibiting factor from calf thymus is described. It seems to fit the definition of a lymphocytic chalone because it is not species-specific and, when incubated with mouse thymic cells, appears to decrease the incorporation of tritiated thymidine. This substance has the ability to diminish skin allograft rejection, graft-versus-host reaction, lymphocyte transformation by phytohemagglutinin, and formation of hemolysin 19 S against sheep erythrocytes. The material seems to be an acidic protein.—Natl Cancer Inst Monogr 38: 135–142, 1973.

TISSUE-SPECIFIC INHIBITORS of the mitotic mechanisms have been demonstrated in a variety of organs—skin *(1)*, melanocytes *(2)*, granulocytes *(3, 4)*, liver *(5)*, kidney *(6)*, and lung *(7)*. These organ-specific species-nonspecific inhibitors which contribute by a feedback mechanism to the control of the homeostasis of a tissue have been named "chalones" by Bullough. It was of great importance to study if chalones exist in lymphoid tissue because these represent a good material for biological investigations.

The studies were first carried out on secondary lymphoid organs by Garcia-Giralt et al. *(8, 9)* who extracted from a secondary lymphoid organ (ox spleen) a substance exhibiting the ability to decrease DNA synthesis by human lymphocytes in established cell lines and inhibit various immunological reactions, and by Jones et al. *(10)* who extracted from pig lymph nodes an inhibitor of lymphocyte transformation in the presence of phytohemagglutinin (PHA).

The purpose of our work has been to investigate the possible presence of an inhibitor in the thymus which is a primary lymphoid organ. Therefore, we prepared a thymic extract according to the method described by Bullough and Laurence *(11)* for the epidermal chalone and also used by Garcia-Giralt for the spleen lymphocyte inhibitor.

MATERIALS AND METHODS

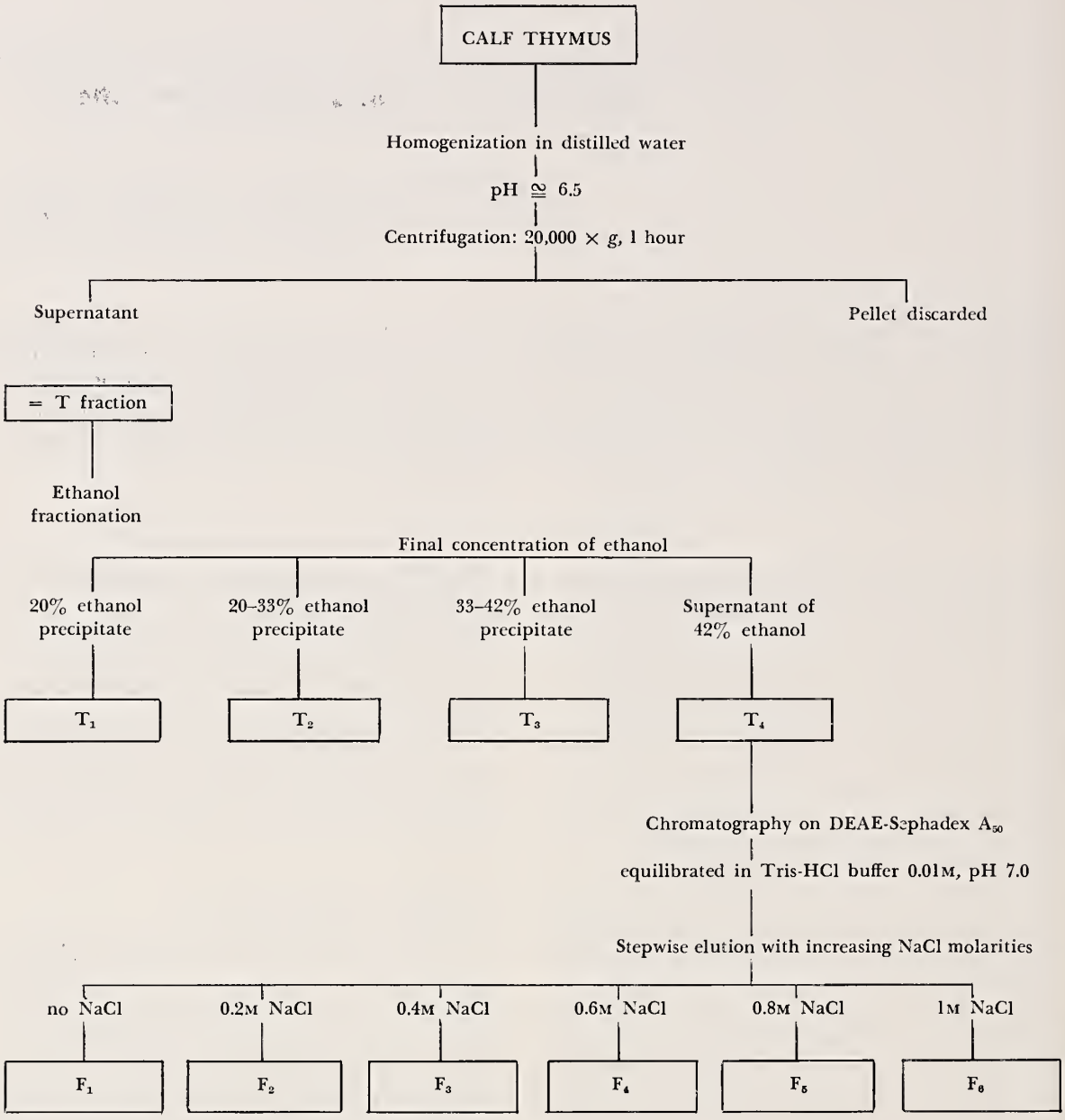
Extraction and purification from calf thymus.—The calf thymus was minced and ground in distilled water and centrifuged for 1 hour at 20,000 × *g*. The supernatant fraction (T fraction) was partially purified by ethanol precipitation. Four fractions were obtained: T_1, T_2, and T_3 which were precipitated, respectively, by final ethanol concentrations of 20, 33, and 42%, and T_4 which was the supernatant of T_3 (chart 1). After dialysis against distilled water and lyophilization, the fractions were stored at −20° C. A fraction, K, corresponding to fraction T, was prepared from calf kidney and was used as a control.

[1] Presented at the First Symposium of the International Chalone Conference, Brook Lodge, Augusta, Mich., June 5–7, 1972.

[2] Supported by grant 66.204 of INSERM (contract DGRST).

[3] We are grateful to Miss Martine Davigny, Mrs. Nicole Verhnes, and Miss Nicole Saou for their excellent technical assistance.

Chart 1.—*Extraction and purification of thymic extracts*



For purification by chromatography, 100 mg of the active fraction was loaded into the top of a DEAE Sephadex A_{50} column equilibrated with 0.01M Tris-HCl buffer at pH 7.0. A stepwise elution of increasing molarity in sodium chloride in the same buffer allowed the separation of 6 fractions: F_1 at 0M, F_2 at 0.2M, F_3 at 0.4M, F_4 at 0.6M, F_5 at 0.8M and F_6 at 1M NaCl.

Analytical electrophoresis was performed on 7% acrylamide gel at pH 9.5 according to the method of Davies *(12)*. The glucidic compounds were demonstrated by staining with periodic acid-fuchsin and the proteins, by staining with amidoblack. Preparative electrophoresis was performed with unfixed gels cut into 5–mm segments which were eluted by homogenizing them in distilled water; the eluate was dialyzed against distilled water.

Tests in vitro.—Rat thymic cells were suspended in RPMI 1640 medium (Eurobio) (5×10^6 cells/ml) containing 10% fetal calf serum and antibiotics. Each fraction to be tested was added at a concentration of 250 μg/ml. PHA (Difco Laboratories), 10 μl reconstituted in 5 ml of isotonic saline, was added. The tubes were incubated for 48 hours at 37° C in a 5% CO_2 atmosphere; 3 μCi of tritriated thymidine (^{3}H-TDR) (s.a., 7 Ci/mmole) was added to each tube 6 hours prior to the end of the culture. The test was terminated by precipitating the acid-insoluble fraction. The radioactivity was counted in a Packard Tri-Carb scintillation counter.

For spontaneous incorporation of ^{3}H-TDR, the fraction to be tested (1 mg in 0.05 ml of saline) was incubated for 3½ hours at 37° C in the presence of 10^7 mouse thymocytes in 4 ml of RPMI 1640. At 30 minutes prior to the end of incubation, 5 μCi of ^{3}H-TDR was added to each tube. The incorporated radioactivity was then determined as described above.

Tests in vivo.—Each group of A/J mice received intraperitoneally 14 mg of protein of the fraction to be tested (T_1, T_2, T_3, T_4) or of the control fraction, K, at the same time as they were given sheep erythrocytes (SRBC). One group received only SRBC. Five days later the number of cells in the spleen producing hemolysin was measured by the test of Jerne and Nordin *(13)*.

In the skin allograft test, C3H tail skin was grafted onto the back of BALB/c recipients on day 0. On days −1, +1, 4, 6, 8, 11, 13, 15, and 19 the mice were given 7 mg of fraction T_1, T_2, or T_4 by intraperitoneal injection. A control group received no extracts.

To study the graft-versus-host reaction (GVH), donors were 3-month-old DBA/2 mice given 2 intraperitoneal injections (with a 6-hour interval between injections) of 0.5 mg of the fraction to be tested. Twenty hours later, 3×10^7 spleen cells of these animals were injected intraperitoneally into 3-month-old (DBA/2 × CBA)F_1 mice previously irradiated with 450 rads total-body irradiation (^{60}Co source). The GVH reaction was estimated from the splenomegaly observed 14 days after the graft.

Also, the donor cells were subjected to in vitro incubation with the fractions. Spleen cells from 3-month-old DBA/2 mice were incubated at 37° C for 1½ hours in RPMI 1640 with the fraction to be tested or with the control fraction (120 μg of protein for 10^7 cells). At the end of the incubation 3×10^7 viable cells were injected intraperitoneally into (DBA/2 × CBA)F_1 mice. The GVH reaction was estimated from the mortality of the animals.

RESULTS

Activity of Crude Fraction After Ethyl Alcohol Precipitation

Text-figure 1 shows that, in the two groups given injections of T_1 and T_4 fractions, there was a signficant decrease in the number of plaque-forming cells in the spleen when compared to the groups receiving K extract or no extract. In contrast, the groups receiving T_2 and T_3 showed no significant differences.

As shown in text-figure 2, repeated injection of T_4 into BALB/c mice moderately but significantly prolonged the survival of C3H skin allografts, compared to controls receiving no extract and to animals given fraction T_1 ($P<0.001$) or fraction T_2 ($P = 0.02$).

Fraction T_4 caused a 96% inhibition of the incorporation of ^{3}H-TDR induced by PHA in rat thymocytes, while control fraction K, pre-

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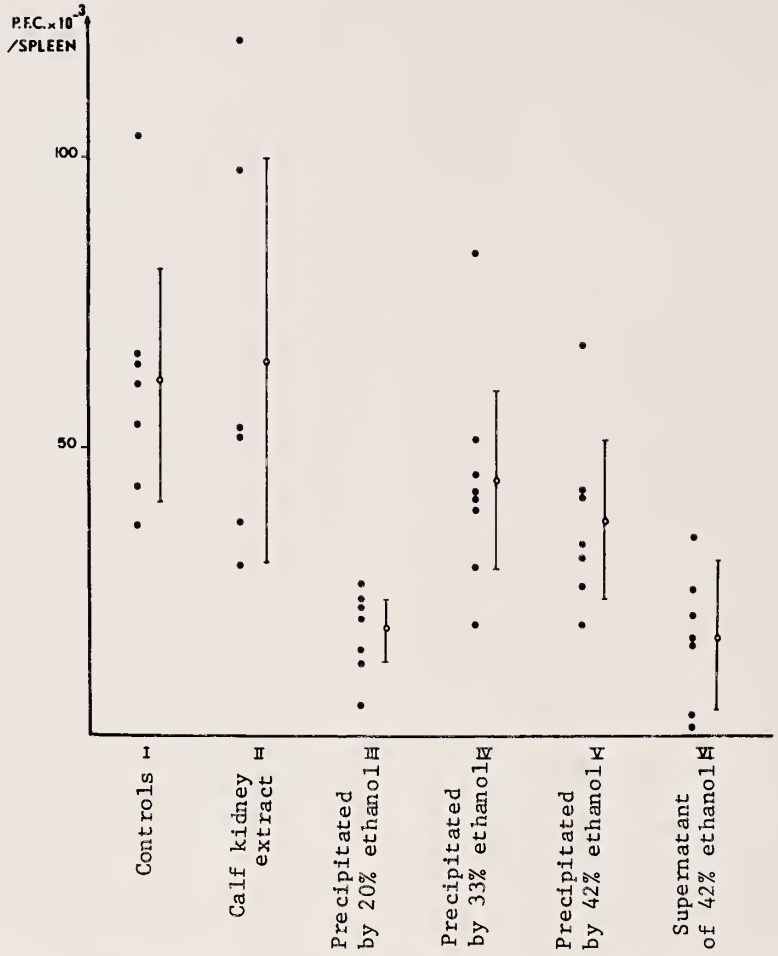


TEXT-FIGURE 1.—Action of various fractions on the formation of hemolysin 19 S against SRBC.

pared from calf kidney, had no effect (text-fig. 3). In all cases, the observed inhibition was not due to a cytotoxic action of T_4.

Incubation with the T_4 fraction decreased the capacity of parental spleen cells to elicit a GVH reaction (text-fig. 4). All animals receiving spleen cells treated with the control fraction K were dead by 27 days after the graft (mean survival time, 15.5 days) while animals receiving spleen cells treated with the T_4 fraction had a mean survival time of 72.3 days.

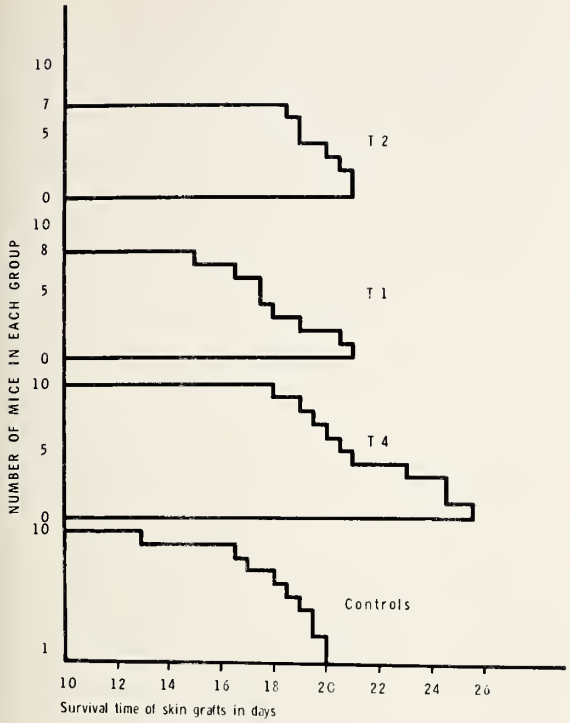
Activity of Subfractions Purified by Chromatography

Among the six purified fractions, only F_4 (eluted at 0.6M NaCl) could produce 96% inhibition of the incorporation of ^{3}H-TDR by mouse thymocytes when 10^7 cells were incubated with 1 mg of F_4 (text-fig. 5).

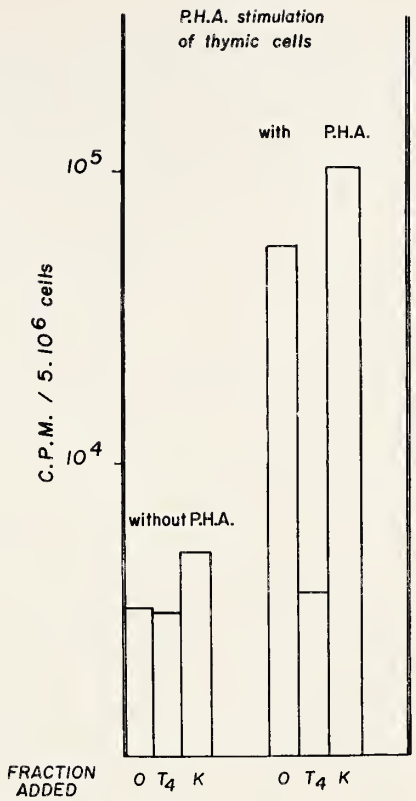
Pretreatment of parental donors with two injections of 0.5 mg of purified fraction F_4 inhibited the capacity of their spleen cells to elicit a GVH reaction (text-fig. 6). The other fractions had no effect on the immune capacities of the donor lymphocytes.

Preliminary Physicochemical Properties of F_4 Fraction

Electrophoresis of 500 mg of F_4 on acrylamide gel showed, on staining with amidoblack, a major band (A, R_f about 0.76) and a minor band (C, R_f 0.94) (fig. 1). Oxidizing the gels with peri-



TEXT-FIGURE 2.—Effect of various fractions on survival of skin allografts.



TEXT-FIGURE 3.—Transformation of thymic lymphocytes by PHA and its inhibition by fraction T_4. Each *column* represents the mean of six determinations.

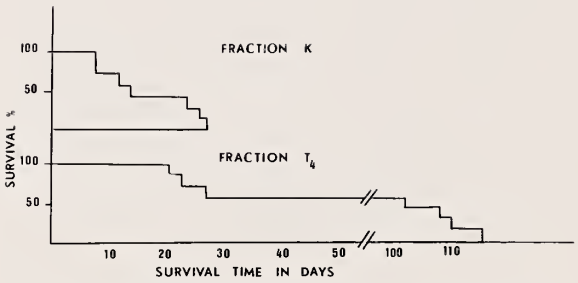
odic acid followed by staining with fuchsin to demonstrate glycoprotein showed an intensely stained band in the C region, a thin band in the A region, and a large diffuse band B between A and C. By preparative electrophoresis, the activity (measured by the effect of the eluate on the spontaneous incorporation of ^{3}H-TDR in mouse thymocytes) was recovered in the area of band A.

DISCUSSION

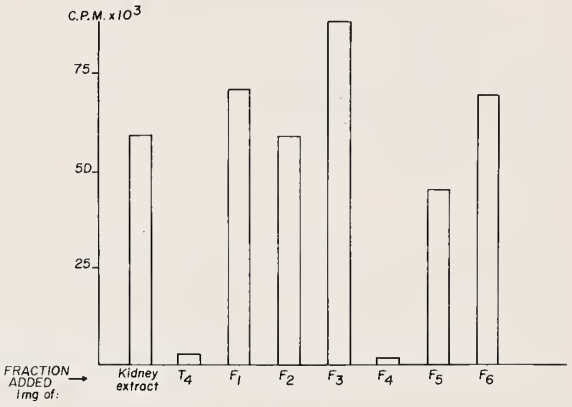
The conditions of elution of the active fraction on DEAE Sephadex A_{50}, as well as its behavior on analytical electrophoresis, suggest that the biological activity is related to an acidic protein. From these observations we can argue against the hypothesis that we have isolated one of the immunosuppressive thymic substances previously described by some workers and identified as histones (*14, 15*) or α_2-glycoproteins (*16*). The same argument can be used to rule out the presence of an hypothetical γ-globulin. However, we cannot say at the present time if our substance is identical with the splenic inhibitor extracted by Garcia-Giralt et al. or if they are two unrelated substances acting by different mechanisms on immune reactions.

The lymphocyte-inhibiting factor we have extracted from the thymus seems to answer the definition of a lymphocytic chalone. It is not species-specific, as shown by the fact that it is extracted from calf thymus (and also from lamb thymus) and acts in the mouse and in the rat. It inhibits lymphocytic proliferation, as shown by the marked decrease of ^{3}H-TDR incorporation by mouse thymocytes in the presence of the active purified fraction.

As a result of the inhibition of this lymphocytic proliferation, we are able to decrease immune

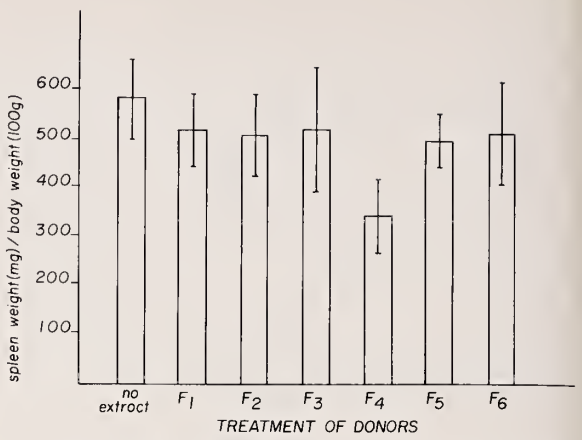


TEXT-FIGURE 4.—Effect of incubation with fraction T_4 on GVH reactivity of parental spleen cells injected into F_1 hybrid recipients.



TEXT-FIGURE 5.—Effect of fractions obtained by chromatography on incorporation of ^{3}H-TDR by mouse thymocytes.

responses including those in which T-lymphocytes are totally involved—such as PHA-lymphocytic transformation (*17*), GVH reaction (*18*), and skin allograft rejection—and one in which T-lymphocytes are involved together with B-lymphocytes—formation of hemolysins against SRBC (*19*). In the case of the PHA-induced transformation, the reaction has been entirely



TEXT-FIGURE 6.—Effect of pretreatment of parental donors on GVH reactivity of their spleen cells injected into F_1 hybrid recipients.

inhibited by addition of our fraction to the culture. The development of splenomegaly normally observed after injection of parental spleen cells into F_1 hybrid recipients has been suppressed by treatment of donors with low doses (two doses of 0.5 mg) of the F_4 purified fraction. Because of its practical interest we have carried out an attempt at in vitro treatment of the donor spleen cells before injecting them into the F_1 hybrid recipients. In this case, too, we observed a significant decrease of the GVH reaction, demonstrated by a considerable delay in the mortality of animals given injections of cells previously incubated with the T_4 fraction.

We have obtained a slight but significant prolongation of skin allograft survival time when high doses of T_4 fraction were repeatedly administered to the recipients. This experiment may be improved by using the more purified material at low doses. Finally, we have observed an inhibition of the formation of hemolysin 19 S against SRBC when animals were given injections of T_4 fraction at the same time as the antigen.

In all these immune reactions, T-lymphocytes are involved. Because we may expect a close specificity of action of a thymic chalone on thymus-dependent lymphocytes, we are now investigating if our extract is able to inhibit immune reactions in which only B-lymphocytes are involved.

It is interesting to note that in the thymus there exist two factors, one permitting the maturation of bone marrow stem cells into T-lymphocytes as indicated by the recent experiments of Goldstein et al. (*20*), Small and Trainin (*21*), and ourselves (*22*), and another controlling the proliferation of lymphocytes, specially T-lymphocytes, and consequently the expression of their immunological functions. Both these factors could play a considerable role in the lymphoid tissue homeostasis.

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FIGURE 1.—Electrophoresis of fraction F_4 on polyacrylamide gel. *Left:* Stained for glycoproteins (periodic acid-fuchsin). *Right:* Stained for glycoproteins, then restained with amidoblack.

Chalone of the Granulocyte System [1, 2]

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SUMMARY—Granulocytic chalone is a tissue-specific, species-nonspecific regulator substance which inhibits DNA synthesis in the granulocyte system. The substance is present in the mature and immature cells of the system, and evidently it is also synthesized within these cells. Natl Cancer Inst Monogr 38: 143–146, 1973.

THERE ARE theoretical reasons for believing that a group of tissue genes exists which may be called "chalone genes" (*1*); these would specify both the structural details and the rate of synthesis of the tissue-specific chalone. Evolution of "chalone genes" in metazoans seems to imply that, at least in principle, each cell is potentially capable of synthesizing chalone and, therefore, that a chalone system exists in every tissue of a mammalian organism, including granulocytes.

Cell recruitment in the granulocyte system is different from that in ordinary tissues: Mitotically active immature granulocytes form a series of transit populations which are maintained only by the continuous feeding of new cells from a pluripotential stem cell compartment [*see* (*2*)]. It is improbable that granulocytic chalone would control this type of induction process, and indeed the activation of "chalone genes" directing the synthesis of a specific granulocytic chalone may itself be the consequence of the induction. It thus follows that, if anything, granulocytic chalone controls the number of mitoses through which each maturing cell passes. If granulocytic chalone acts by preventing immature cells from entering the generative cell cycle, it is possible that it also accelerates the maturation rate of the cells (*3, 4*).

This article provides a brief summary of what is now known of the granulocytic chalone and of its mode of action. The chemistry of this substance is not dealt with here; it is summarized by Paukovits in the next paper (*5*).

TISSUE SPECIFICITY

Tissue specificity is the most important characteristic of chalones but unfortunately it is also a difficult problem to study. The lack of extensive evidence of this property is perhaps one of the main reasons why the existence of most of the 10 or 11 chalones suggested in the literature has not been generally accepted [*see* (*6, 7*)]. With respect to the granulocytic chalone, evidence for tissue specificity is based on the following findings.

It has been demonstrated (*8*), by means of autoradiographic analysis of rat bone marrow cells labeled with thymidine-^{3}H (^{3}H-TDR) in a short-term in vitro culture, that the number of labeled granulocytic cells was significantly decreased in the presence of an unpurified preparation of granulocytic chalone. In contrast, no change could be observed in the labeling index of the other cell types of the bone marrow. This inhibitory effect cannot be explained in terms

[1] Presented at the First Symposium of the International Chalone Conference, Brook Lodge, Augusta, Mich., June 5–7, 1972.

[2] Work summarized here was supported by grants from the Lady Tata Memorial Trust, London, and from the Sigrid Jusélius Foundation, Helsinki.

TABLE 1.—*Fractionation of bone marrow and liver extracts on Sephadex G-25**

Fraction No.	Bone marrow extract		Liver extract	
0	111±3	111±4	102±4	92±6
1	103±3	94±6	90±6	94±2
2	104±2	92±1	96±5	87±3
3	101±2	83±1	95±9	95±3
4	105±2	111±3	126±3	98±6
5	85±4	107±6	91±8	90±4
6	104±3	111±12	86±3	98±1
7	110±5	117±3	98±7	105±4
8 Expected	81±6	95±2	102±3	109±6
9 elution zone	65±3	71±2	108±6	111±6
10 of chalone†	84±5	85±2	119±6	101±5
11	101±5	99±8	116±7	105±5
12	115±4	104±7	105±5	103±3
13	112±6	100±5	87±2	107±8
14	108±2	116±18	85±1	101±3
15	113±4	105±3	94±2	104±4

*Effects of the fractions were measured in terms of total incorporation of ^{3}H-TDR in rat bone marrow cells in vitro. The counts recorded are expressed in relation to the mean incorporation within each time group (culture time).

†Location of this zone was based on earlier findings [*see* (*13*)].

of selective toxicity of the test preparation on immature granulocytes, because the number of granulocytic cells is not influenced by chalone in short-term cultures (*4, 8, 9*) and because the inhibition so produced is reversible (*4, 9*).

New evidence for the cell-line-specific action of the granulocytic chalone has been obtained recently by means of a closed in vivo culture system utilizing diffusion chambers (*10*). This technique has several advantages as compared to the standard in vitro bone marrow culture which we have used previously (*8, 11*). In the diffusion chamber cultures in particular, immature cells generate granulocytes at a rate comparable to that found in granulopoietically stimulated bone marrow in situ (*12*)—i.e., the cell proliferation rate is much faster than that found in the in vitro assay (*4*). Diffusion chamber experiments, in which the tissue specificity of the granulocytic chalone was assessed, were performed as follows.

Chalone (obtained from rat bone marrow granulocytes) and control solutions (obtained from rat liver) were first subjected to gel filtration on Sephadex G-25 and the fractions collected were tested by means of the standard in vitro assay technique [*see* (*8, 11*)]. The results (table 1) showed that an inhibitor of DNA synthesis was present in the test solution and that the elution of this inhibitor corresponded to the expected elution zone of granulocytic chalone [*see* (*13*)]. A similar inhibitor was not present in the control solution prepared from rat liver.

Chalone and control solutions were then injected into mice carrying two different kinds of 5-day chamber cultures in the peritoneal cavity. One chamber contained mouse bone marrow cells—proliferating granulocytes and macrophages (*14*)—and the other chamber contained mononuclear cells isolated from rat blood—proliferating immunoblasts and macrophages (*15*). Three hours after two intraperitoneal injections of chalone or control solutions, the mice were given injections of ^{3}H-TDR and the incorporation of label into the chamber cells was measured by means of liquid scintillation counting. The results obtained in three separate experiments are summarized in table 2.

The chalone preparation tested caused 26% inhibition of the incorporation of ^{3}H-TDR in proliferating granulocytes. (In these cultures, macrophages did not contribute much to the total ^{3}H-TDR uptake, because their labeling index was low and their total number was about the same as the number of proliferating granulocytes.) In contrast to this inhibition, no effect was observed on the chamber cultures containing proliferating immunoblasts and macro-

TABLE 2.—*Effect of (rat) granulocytic chalone on proliferating granulocytes, macrophages, and immunoblasts in closed in vivo culture (diffusion chamber technique)**

Target cells (chamber culture)	Solution injected	Number of chambers studied	Number of cells harvested per cell inoculated	Incorporation of ^{3}H-TDR per 10^6 cells (% of injected dose)	Inhibitory effect (%)
Mouse granulocytes and macrophages	Chalone	14	1.31±0.12	0.130±0.017	26
	Control	15	1.32±0.12	0.175±0.017	
Rat immunoblasts and macrophages	Chalone	10	0.60±0.06	0.111±0.027	1
	Control	13	0.53±0.05	0.112±0.017	

*Compiled from data of Benestad, Rytömaa, and Kivilniemi (*10*).

phages. Furthermore, the viability of the cultured cells was apparently not influenced by the chalone treatment, because the cell yields were about the same in the test and control groups.

In this set of three experiments the chalone effect was statistically significant at the level, $P<0.05$ (Student's t test), which may not appear very impressive. It must be noted that, in two other sets of diffusion chamber experiments in which different batches of granulocytic chalone were tested on proliferating granulocytes (and macrophages), the inhibitions observed were 18% and 66% ($P<0.001$), respectively (*10*). It thus follows that the chalone action on ^{3}H-TDR uptake in proliferating granulocytes is real.

In addition to these two main lines of investigation of the tissue specificity of the granulocytic chalone, further supporting evidence has been obtained in vivo and in vitro. It was found that prolonged, intensive treatment of chloroleukemic rats with granulocytic chalone depressed leukemia cell proliferation to a great extent (with subsequent disappearance of all malignant cells from some animals by some supporting, probably immunological, mechanism), but that this treatment did not result in any distinct inhibition of cell proliferation in nongranulocytic cell lines (*16–18*). Another small piece of evidence for the specificity of the granulocytic chalone may also be mentioned in this context: When a crude preparation, effective in our standard in vitro assay, was tested on the mitotic activity of the epidermal cells in vitro, no effect could be seen (*19*).

GRANULOCYTIC CHALONE AND THYMIDINE METABOLISM

The inhibitory effect of the granulocytic chalone has usually been studied by measuring ^{3}H-TDR incorporation in the target cells. It may therefore appear possible that the effect of the test preparations could simply be explained in terms of interaction with thymidine metabolism—e.g., in terms of a factor diluting exogeneous ^{3}H-TDR. This is evidently not the case, as is indicated by the following findings.

1) Inhibition by chalone of DNA synthesis in normal rat bone marrow cells and in chloroleukemic cells in vitro is readily demonstrable by means of ^{14}C-labeled formate (*9, 11*). This excludes pseudoinhibitory mechanisms such as enhanced conversion of thymidine to thymine or inhibition of the reaction step from TDR to TMP, both of which would decrease uptake of ^{3}H-TDR into DNA.

2) It was shown above that the action of the granulocytic chalone is tissue-specific, both in the standard in vitro assay and in the closed in vivo culture. It is thus most improbable that the effect of the test preparation could be based on a direct, nonspecific interaction with thymidine metabolism.

Despite this and other similar evidence (*8*) for real inhibition of DNA synthesis in the immature granulocytes, it may also be important to demonstrate directly that granulocytic chalone decreases the production of mature granulocytes. So far, good experimental evidence of this parameter is not available. In principle, the diffusion chamber technique appears to provide a well-controlled assay system to reveal such an effect. It may be worth noting, however, that chalone treatment must be started early in the growth phase of the chamber cells; otherwise a possible prolongation of life expectancy of the cells [*see* (*1*)] could prevent significant alteration

of the population size. Such an outcome would be analogous to the situation observed by Bullough and Ebling (*20*): Although these authors maintained the epidermal mitotic rate of adult mice at a quarter of its normal level for 4 weeks (by means of a long-continued stress), no change was found in either epidermal thickness or sebaceous gland size.

SUMMARY OF PROPERTIES OF GRANULOCYTIC CHALONE

The following list summarizes the main biological characteristics of the granulocytic chalone revealed thus far by the studies of Rytömaa and Kiviniemi (*4, 8, 9, 11, 16–18*), Paukovits (*21*), and Benestad, Rytömaa, and Kiviniemi (*10*). 1) The substance is produced by granulocytic cells. 2) It inhibits DNA synthesis of proliferating granulocytic cells (both normal and leukemic). 3) This inhibition is cell-line-specific but not species-specific. 4) Inhibition of DNA synthesis is short-term and reversible.

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Granulopoiesis-Inhibiting Factor: Demonstration and Preliminary Chemical and Biological Characterization of a Specific Polypeptide (Chalone) [1]

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SUMMARY—A polypeptide with granulopoiesis-inhibiting properties has been isolated from bone marrow-conditioned medium and from spleen supernatant fraction. The preparation involves ultrafiltration, gel chromatography, and paper electrophoresis. This polypeptide inhibits the proliferation of the myeloid precursor cells in the bone marrow. The action on the different cell types of the myeloid series was demonstrated in a double-labeling experiment involving separation of the bone marrow cells by ficoll density step centrifugation and velocity sedimentation. The results indicate that this polypeptide may be identical with the granulocytic chalone.—Natl Cancer Inst Monogr 38: 147–155, 1973.

THE MAINTENANCE of regularly balanced conditions in the production system of blood cells, as well as the adjustment of the number of circulating cells to functional demands, requires the action of specific feedback circuits between the bone marrow and peripheral "sensors" which monitor the functional state of the whole system and correspondingly regulate the output from the producing compartments. These feedback loops control either the rate of stem cell transformation to blast cells or the rate of subsequent mitotic divisions and differentiation processes. The peripheral sensors should be responsive to either the functional capacity or the mass of circulating cells.

It is obvious that the factors which regulate the production rate in the bone marrow, as the result of some at present largely unknown sensor action, should be of humoral nature. Several such regulatory substances have been detected and characterized during the past few years. It appears that generally, but certainly oversimplified, the output from the proliferative compartments of the bone marrow is regulated by the concerted action of stimulating and inhibiting substances. In the erythropoietic system this regulation is exerted by erythropoietin and an erythropoiesis-inhibiting factor (EIF) (*1*) which may be identical with the proposed erythrocytic chalone (*2*). The rate of granulopoiesis is similarly controlled by a granulopoietin (colony-stimulating factor) (*3, 4*) and the action of a granulopoiesis-inhibiting factor (GIF) (*5, 6*) which may be identical with the proposed granulocytic chalone, due to its specific feedback effect.

Chalones have been defined as being specific hormone-like regulators which are produced by a tissue and which regulate the functional and mitotic homeostasis of this same tissue by inhibiting cell division and promoting differentiation. A further property is the apparent lack of species specificity as far as the biological action is

[1] Presented at the First Symposium of the International Chalone Conference, Brook Lodge, Augusta, Mich., June 5–7, 1972.

[2] I gratefully acknowledge the skillful technical assistance of Mrs. J. Paukovits.

concerned. Whether this implies a chemical identity among different animal species or not is at present unknown. It may well be that certain smaller interspecific structural differences exist, as are observed in many polypeptide hormones.

At present, knowledge about the chemistry of the chalones and their mechanism of action is scanty. It is known that several tissue extracts specifically inhibit mitosis in the respective homologous tissue in a characteristic manner which may be attributed to the action of a chalone. These extracts have no influence on the metabolic rate of DNA synthesis or on the speed at which the cells pass through the stages of the generative cycle. The implication thus is that chalone may act on an unknown switch mechanism in the G_1 phase, which determines whether a cell will go into DNA synthesis and subsequent mitosis or will remain mitotically inactive.

Until now, despite considerable effort, no really successful work has been reported which definitely shows that any chalone exists as a chemically definable substance. It should be noted, however, that in many experiments in which some chromatography has been performed, the purification of the respective chalone was not the most important point. It appears that really meaningful results about the existence of the chalones, their nature, and their mechanism of action cannot be obtained unless at least partially purified preparations are used, in which the impurities have no disturbing biological activity.

In this paper, methods for the preparation of highly purified GIF and some experiments concerned with the specificity of the biological action of this substance are described.

MATERIALS AND METHODS

Bone marrow-conditioned medium.—Bone marrow cells from the femora of 2-month-old female rats were incubated for 5 hours at 37° C in a medium containing amino acids dissolved in Hanks' balanced salt solution (BSS) (the amino acid concentrations were the same as in medium 199). The medium also contained 0.2 μCi/ml of ^{14}C-labeled protein hydrolysate from chlorella (The Radiochemical Centre, Amersham). In some experiments, ^{3}H-leucine (Amersham) was used (1 μCi/ml). The incorporation of radioactively labeled leucine into peptide material was increased by omitting unlabeled leucine from the amino acid mixture. For incubation, 50 ml of this medium was used for the bone marrow cells obtained from the femora of 4 rats. After incubation the cells were removed by centrifugation at 700 $\times$ *g* for 10 minutes and the supernatant was collected. Usually, the conditioned media from 40–50 femora were pooled and used for further work.

Spleen extract.—A total of 40–50 rat spleens were homogenized at 0° C (5 ml of distilled water for 2 spleens). The resulting suspension was centrifuged at 50,000$\times g$ for 45 minutes and the clear supernatant was used for further work.

Ultrafiltration.—The supernatants from bone marrow or spleen were concentrated by ultrafiltration at 0° C. All large proteins were first removed by filtration through Amicon UM–10 membranes (exclusion limit, 10,000 Daltons). The filtrate from this step was then concentrated, and small molecules were partly removed by filtration through an Amicon UM–05 filter (exclusion limit, 500 Daltons). The residue from this step was used for further work. In some cases this concentration was performed by rotary evaporation.

Column chromatography.—Columns of Sephadex G-25 (3 $\times$ 50 cm or 3 $\times$ 140 cm) were eluted with either 1M acetic acid or 0.01M phosphate buffer (pH 7.4). The columns were designed for minimal dead volume (0.1 ml) at the lower end. The effluent was monitored at 280 nm, and fractions were collected. The radioactivity was measured in a 100-μl aliquot of each fraction by using a dioxane-based scintillation liquid. Gel chromatography on columns of Sephadex G–15 was performed with distilled water as eluant. All chromatographic procedures were carried out at room temperature. A 100- to 500-μl sample of each fraction was examined in the bioassay screening system after careful evaporation of the acetic acid.

Paper electrophoresis.—Electrophoresis was performed on Whatman 3MM paper. The elec-

trolyte solution was acetic acid-formic acid-water, 78:25:888 (v/v/v). Electrophoresis was run for 3 hours at 7.5 v/cm without cooling. The increase in current during the run was within 10% of the initial value. All papers were prewashed by prolonged "chromatography" with 1-butanol-acetic acid-water, 4:1:2 (v/v/v).

Radioactively labeled substances were detected on the paper by cutting the dried paper into sections, 5–10 mm wide, and measuring the radioactivity of each strip in the liquid scintillation counter with a toluene-based scintillation fluid. After counting, the strips were washed several times with toluene to remove the scintillation chemicals. Peak fractions were subsequently eluted with 20% acetic acid overnight.

In the experiments with nonlabeled material, the peptides were visualized by dipping guide strips in ninhydrin reagent (1 g of ninhydrin in acetone-acetic acid-water, 100:5:10, v/v/v) and heating for 5 minutes at 90° C. In some cases, the reflectivity at 570 nm of the ninhydrin-stained strips was recorded using a Zeiss PMQ II Spectrophotometer equipped with a chromatogram scanner.

Thin-layer chromatography.—Ascending thin-layer chromatography was performed on sheets precoated with microcrystalline cellulose (Polygram CEL 400, Macherey and Nagel, Germany). The solvent systems used were 1-butanol-acetic acid-water 4:1:2 (v/v/v), and 1-butanol-pyridine-acetic acid-water, 75:50:15:60 (v/v/v/v). In the preparative mode, only the second solvent system was used and the zones corresponding to ninhydrin-stained guide spots were scraped off. The substances were eluted overnight with 20% acetic acid and the acid was removed by rotary evaporation after centrifugation of the cellulose particles.

Amino acid analysis.—The substances eluted from thin-layer sheets were hydrolyzed overnight at 105° C with 5.7N HCl in a nitrogen atmosphere. Amino acid analysis was performed on a Beckman Unichrom amino acid analyzer by the single-column technique. The analyzer was equipped with a 10-mm cuvette and a 1-mV range card on the recorder, thus permitting the analysis of molar amounts.

Screening of chromatographic fractions for inhibitory activity.—The biological activity of the fractions obtained from column chromatography or from eluted paper strips was determined by measuring the decrease in incorporation of thymidine-^{3}H into rat bone marrow cells in vitro. A detailed description of the method has been published (*6*).

Preparation of immature granulocytic cells.—Mitotically competent immature myeloid cells were prepared by the method of Evans et al. (*7*). The separation procedure involves the centrifugation of bone marrow cells through a dense ficoll solution. A layer of cells, consisting mainly of blasts, promyelocytes, and myelocytes, is obtained at the interface of the starting sample layer and the ficoll solution (FT-layer).

Velocity sedimentation.—The bone marrow of the femora and tibiae of 3–5 guinea pigs (400 to 500 g) was removed and the myeloid precursor cells were prepared according to Evans et al. (*7*). The cells of the FT-layer were suspended in 20% calf serum in Hanks' BSS and this suspension was divided into two portions. The cells of the first portion were incubated in the presence of thymidine-^{3}H (50 μCi/ml) and GIF, whereas the cells of the second portion were incubated with thymidine-^{14}C (5 μCi/ml). After 5 hours at 37° C, the cells were washed free of the 20% medium and resuspended in 5% medium. Then, the two portions were combined and the cell number was adjusted to 2.5×10^{6}/ml.

A sedimentation chamber constructed according to Miller and Phillips (*8*), with conical bottom and 5–cm diameter, was kept at 8° C. It was filled from the bottom at a rate of 200 ml/hour with 4 ml of phosphate-buffered saline, 4 ml of the combined cell suspension (a total of 10^{7} cells), and a linear gradient from 15 to 30% calf serum in Hanks' BSS. The function of this gradient is solely to decrease thermal convection in the chamber. After a sedimentation time of 3–4 hours, the chamber was emptied and 35 4–ml fractions were collected. The amount of ^{3}H and ^{14}C activity in the cells of each fraction was measured in the trichloroacetic acid- and methanol-insoluble material as described earlier (*6*). The ratio of radioactivities, ^{3}H/^{14}C, was determined in each fraction by liquid scintillation

counting using the external standard method for calibration.

RESULTS AND DISCUSSION

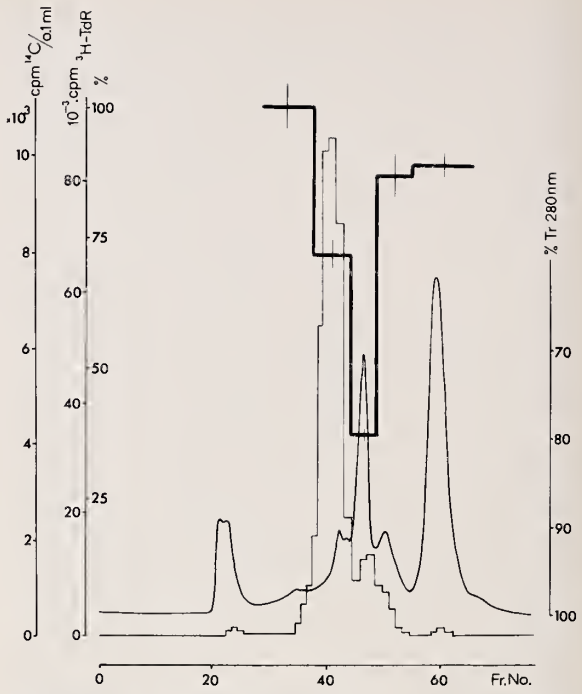
Purification of GIF

Attempts at further purification of the GIF-containing material eluted from Sephadex G–25 columns with phosphate buffer (*6*) led to the conclusion that it would be extremely difficult to desalt the preparations so obtained. Since the elimination of salts is necessary before further purification steps can be successfully applied, we tried to avoid this problem from the beginning by using volatile buffers for gel chromatography. Preliminary results suggested that GIF is relatively stable at low pH. Therefore, 1M acetic acid was used as eluant for the Sephadex G–25 columns which could be run at room temperature without significant losses in the overall yield of inhibitory activity of GIF.

The elution was monitored and the fractions obtained were appropriately pooled and repeatedly lyophilized to remove all acetic acid which otherwise would interfere with the bioassay. The results of a typical experiment are given in text-figure 1. It is easily possible to compare the elution characteristics of GIF from these columns with the results obtained with phosphate-eluted columns (*6*).

The GIF region also contains a large amount of free amino acids which can be separated analytically by several methods including thin-layer chromatography and paper electrophoresis. This amino acid contamination is probably due to considerable irregularities in the elution characteristics of amino acids and peptides from Sephadex G–25.

Further purification of GIF could be achieved by preparative paper electrophoresis at pH 1.9. Under these conditions, amino acids are readily separated from larger molecules. Furthermore, the substance(s) present in the GIF region are effectively desalted. The amount of substance obtained by eluting the zone designated P in text-figure 2 is very low even at the highest tolerable load on the paper. It seems to be possible to carry out some further chemical work by application of purely radiochemical methods.



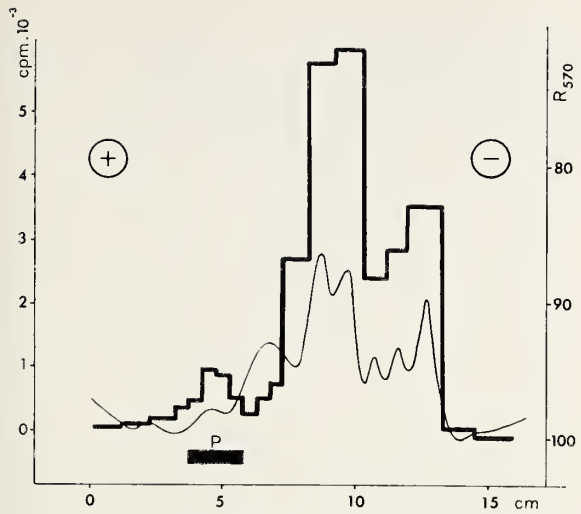
TEXT-FIGURE 1.—Chromatography of rat bone marrow-conditioned medium from 50 femora on a Sephadex G-25 (fine, column 3 by 50 cm). Solvent, 1M acetic acid. Fraction size, 4 ml. *Continuous line:* % transmittance at 280 nm; *thin step-line:* cpm ^{14}C in 100-μl aliquots of each fraction; *heavy step-line:* effect on thymidine-^{3}H (^{3}H-TDR) incorporation into rat bone marrow of 100-μl aliquots of each fraction.

However, it has turned out to be difficult to show that region P of text-figure 2 actually contains material with bone marrow-inhibiting activity. Nevertheless, the only region in the electropherogram which contained GIF activity (although not significant) was P.

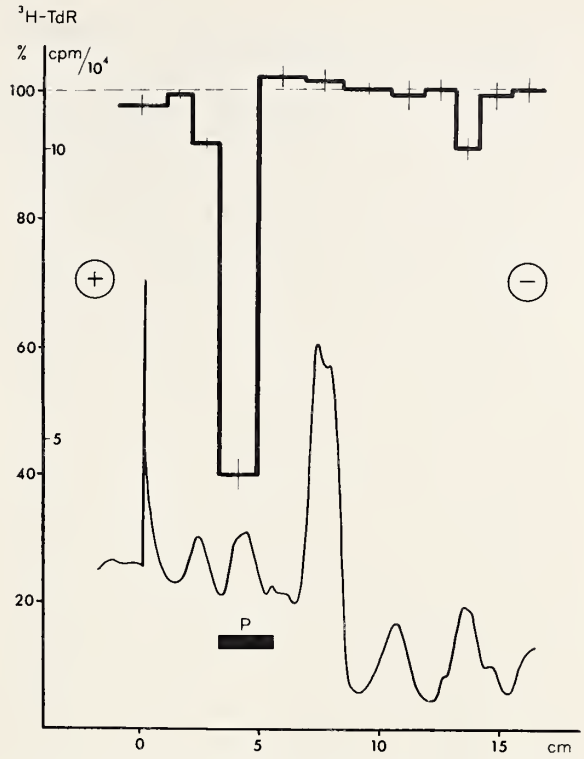
The problem thus arises to find a source of GIF-containing material which is readily available in relatively high amounts.

Isolation of GIF From Supernatants of Spleen Homogenates

Since GIF is actively produced by mature granulocytes, one should expect that organs which contain large numbers of granulocytes should also contain GIF in considerable amounts. We therefore used the spleen as a source of GIF.



TEXT-FIGURE 2.—Paper electrophoresis at pH 1.9 of biologically active region of Sephadex G-25/1M acetic acid-chromatogram (bone marrow-conditioned medium); Whatman 3MM paper, 7.5 V/cm, 3 hours. *Continuous line:* reflectivity at 570 nm after staining of a guide strip; *step-line:* cpm ^{14}C in indicated sections of guide strip. The main portions of the paper were eluted with 20% acetic acid and the biological activity was determined. Only region P contained inhibitory activity, and this was insignificant in amount.



TEXT-FIGURE 3.—Paper electrophoresis of the biologically active region of a Sephadex G-25/1M acetic acid chromatogram (rat spleen homogenate). Conditions of electrophoresis, staining, and elution were as described in text-figure 2. *Continuous line:* reflectivity at 570 nm after staining with ninhydrin; *step-line:* effect of eluted transverse strips on thymidine-^3H incorporation into rat bone marrow.

The isolation of GIF from tissue homogenates which were prepared with the use of water as solvent would yield G–25 pools with a lower salt content than the Hanks' BSS containing conditioned media, thus allowing the application of higher amounts of substance on Whatman 3MM paper.

Supernatants from homogenized rat spleens were used as starting material for the same separation procedures as described above. (Unfortunately, it is not possible to obtain radioactively labeled GIF preparations in this way.) The aqueous spleen supernatant was ultrafiltered through a filter with a 10,000–Dalton exclusion limit. The filtrate was concentrated by rotary evaporation and chromatographed on Sephadex G–25 with 1M acetic acid as described above. The fractions in the GIF region were appropriately pooled, lyophilized, and subjected to paper electrophoresis at pH 1.9 as described above for bone marrow material. The paper was cut transversely into strips according to the ninhydrin color of the guide strips, and the peptides were eluted with 20% acetic acid overnight. The eluates were lyophilized and the bone marrow-inhibiting activity was determined. The results of a representative run are given in text-figure 3.

In the same region P as in text-figure 2, chemically detectable amounts of ninhydrin-positive substance(s) were found and were highly active in inhibiting thymidine incorporation in bone marrow cells. Co-electrophoresis of the eluted P-region material from bone marrow (labeled) and spleen (unlabeled) revealed that the P regions coincide exactly. This leads to the conclusion that the substances contained in these fractions may be identical at least in part.

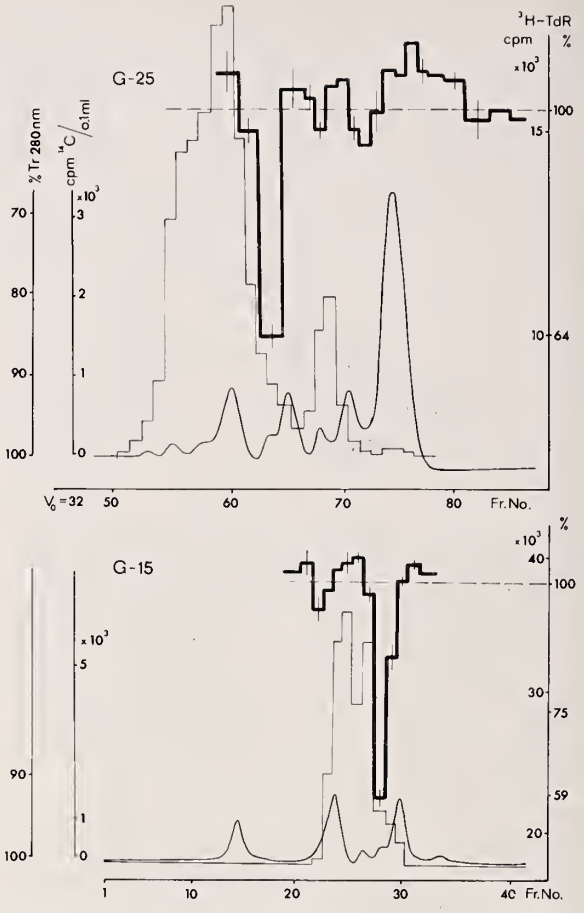
Separation and Preliminary Characterization of Constituents of Splenic Region P

Two-dimensional thin-layer chromatography, on cellulose-coated sheets, of the material eluted from the spleen P region showed the presence of 3–4 ninhydrin-positive spots which could be resolved in one dimension by the solvent system 1–butanol-pyridine-acetic acid-water, 75:50:15:60 (v/v/v/v). The sections of a chromatogram corresponding to the colored regions of a sprayed guide strip were scraped off and the peptides were eluted with 20% acetic acid. No monomeric amino acids were present in the unhydrolyzed eluates, as could be shown by dansylation and two-dimensional thin-layer chromatography. The residues of the eluates were then hydrolyzed and the amino acid composition was determined. No exact values could be determined, due to the exceedingly small amount of material available for analysis. However, the four peptides present in the splenic P region have "normal" amino acid composition. Because one of the four peptides should be the GIF peptide, one could thus conclude that GIF may have an amino acid composition with no striking peculiarities.

Biological Specificity and Determination of Target Cell(s) of GIF

The experiments aimed at purification of the granulopoietic factor demonstrated that the biologically active Sephadex G–25 fractions may contain only one bone marrow-inhibiting substance. It thus was possible to use desalted G–25 fractions for a preliminary determination of the biological specificity of the GIF. In order to avoid the difficulties which arise in the bioassay due to the presence of acetic acid traces, the Sephadex G–25 columns were eluted with 0.01M phosphate buffer, pH 7.4. The elution was monitored as described and 10–ml fractions were collected. The bone marrow-inhibiting activity of each fraction was determined. Bone marrow-conditioned medium (ultrafiltered to contain only molecules below 10,000 Daltons) was used as starting material.

The fractions containing inhibitory material were combined and rechromatographed on Sephadex G–15 columns with water as eluant. The biologically active fractions were again combined and lyophilized. The results of this partial purification are shown in text-figure 4.



TEXT-FIGURE 4.—*Upper:* chromatography of rat bone marrow-conditioned medium from femora on Sephadex G-25 column (fine, 3 by 40 cm). Solvent: 0.01M phosphate buffer, pH 7.4. Fraction size: 10 ml. *Lower:* chromatography of combined biologically active G-25 fractions on Sephadex G-15 (1.5 by 140 cm). Eluant: water. Fraction size: 10 ml. *Continuous line:* % transmittance at 280 nm; *thin step-line*: cpm ^{14}C in 100-μl aliquots of each fraction; *heavy step-line:* effect on thymidine-^{3}H incorporation into rat bone marrow of 500-μl aliquot of each fraction.

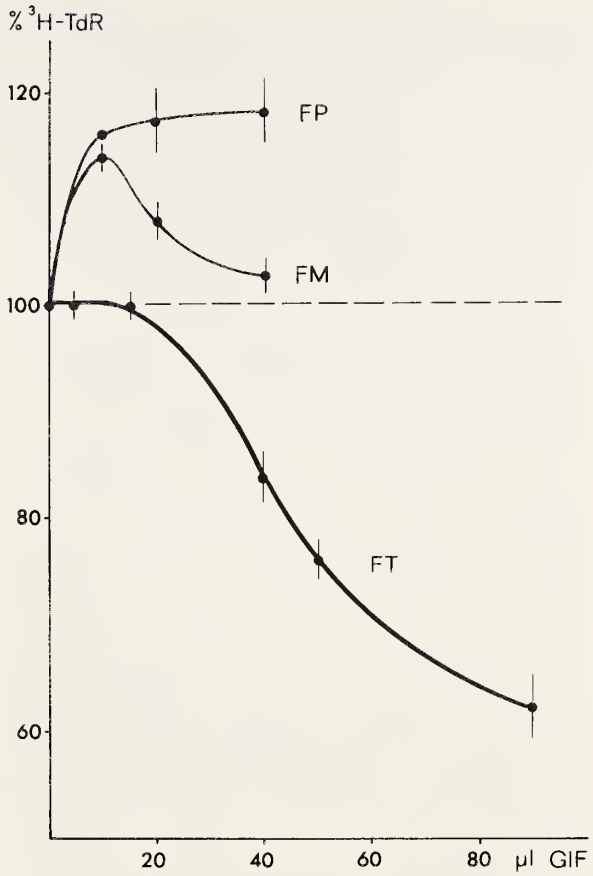
The material obtained in this way caused a 40–50% inhibition of the thymidine incorporation into rat bone marrow. This is indicative of a certain degree of cell specificity and conforms to results of Rytömaa (*9*) which show that bone

marrow-inhibiting material from essentially the same source and with the same elution characteristics as our GIF preparations act specifically by inhibiting the proliferation of granulocytic precursor cells in the bone marrow.

We then tried to determine, by means of physical separation of the myeloid cells of the bone marrow, which particular cell type(s) of the granulocytic series are inhibited by GIF. Myeloid precursor cells of the bone marrow were isolated by the ficoll density step centrifugation method of Evans et al. (*7*). These cells were then further separated by means of velocity sedimentation (*8*). The ficoll step yielded a good enrichment of myeloid precursor cells and an almost complete separation from other cell types in the bone marrow. By the velocity sedimentation step it was possible to subfractionate these myeloic precursors into myeloblasts, promyelocytes, and myelocytes. The in vitro colony-forming units are clearly separated from these cells due to their rapid sedimentation (*10*).

Guinea pig bone marrow was fractionated according to the procedure of Evans et al. (*7*), and three fractions (FT, FM, FP—the top, middle, and pellet layers, respectively) were finally obtained. Of these, FT contained up to 80% of mitotically competent myeloblasts, promyelocytes, and myelocytes. These cells were fully viable and especially were able to incorporate thymidine-^{3}H in a regular manner (*7*). Thus, they were used in the bioassay system instead of whole bone marrow cell suspensions. The effect of increasing amounts of GIF on the incorporation of thymidine into FT, FM, and FP cells is shown in text-figure 5. Only the FT fraction, which contained almost pure granulocytic precursor cells, was inhibited by GIF.

The dose-response relationship of GIF on the whole population of granulocytic precursors has a sigmoid shape. This could be interpreted by assuming an interaction of GIF with a receptor site(s) on the target cells. This sigmoid shape is in accordance with the results of Rytömaa et al. (*5*). It should be noted that the action of several other feedback inhibitors of cell division (chalones) seems to involve also some type of receptor mechanism (*11, 12*).



TEXT-FIGURE 5.—Effect of GIF on thymidine-^{3}H incorporation into subpopulations of guinea pig bone marrow separated by ficoll density step centrifugation. FT is fraction with the highest enrichment of myeloid precursors. The amount of GIF added to the cultures is given in arbitrary units.

In order to determine which one of the myeloid precursor cells is the GIF-responsive cell, the FT population was subjected to velocity sedimentation for further separation of the constituent cell types, as described under "Materials and Methods." The overall sedimentation pattern obtained by this technique is highly reproducible, even in small details. However, it is not possible to compare consecutive runs directly, fraction by fraction. This is mainly due to small differences in the experimental conditions of each run. We thus decided to perform the control experiment and the GIF run simultaneously by using a double-labeling technique. Controls were

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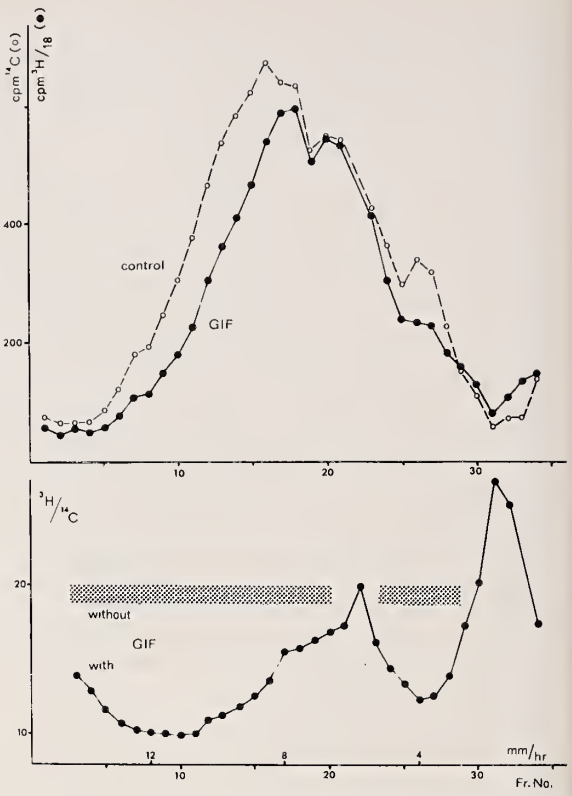
incubated with thymidine-^{14}C, and the test cells were incubated with thymidine-^{3}H plus GIF.

The amounts of radioactivity used in these experiments are very high and actually lie in the range used in the thymidine-suicide technique. The reason for this choice was mainly that only very few cells (10×10^6) could be applied to our STAPUT-chamber for velocity sedimentation. Higher cell numbers inevitably caused the formation of streamers as described by Miller and Phillips (*8*). However, the results obtained should not be influenced by these suicide conditions because the cells go through only one cycle of DNA synthesis during our incubation time.

After incubation, the cells of both suspensions are combined, and a velocity sedimentation is performed with this combined population. Cells which are inhibited by GIF would show up as minima in a plot of $^{3}H/^{14}C$ versus migration distance. Test runs without GIF in both incubation fractions gave (in this system) an isotope ratio of $^{3}H/^{14}C = 18–20$ over the whole gradient. The results show that the DNA synthesis is inhibited by GIF in essentially the whole range of granulopoietic precursor cells (text-fig. 6). Two pronounced minima occurred in the isotope ratio curve, at 4 mm/hour and 11 mm/hour, respectively.

The sedimentation rates of the different cell types turned out to be somewhat higher in our system than those reported in the literature (*10, 13*) but the correlation can easily be made by taking into account the decreased viscosity of the sedimentation medium due to the higher temperature in our system. It is thus possible to identify the cells in the 4 mm/hour region as blasts (myeloblasts or promyelocytes). In the myelocyte range (6–8 mm/hr in our system), only weak inhibition was observed which, taken by itself, is not very indicative of an inhibitory action of GIF on myelocytes. It should be pointed out, however, that cell cycle kinetic effects should play an important role here and that myelocytes are not very active in DNA synthesis. The assumption that GIF also inhibits the proliferation of myelocytes also would be in accordance with the magnitude of the effect of GIF on the whole bone marrow population. This point, however, awaits further clarification.



TEXT-FIGURE 6.—Separation of myeloid precursors (FT cells) by velocity sedimentation. Experimental conditions are given under "Materials and Methods." *Upper:* incorporation of labeled thymidine into control cells (^{14}C) and under the influence of GIF (^{3}H). The ^{3}H-curve is normalized to 1/18, according to the results of a control experiment. *Lower:* isotope ratio, $^{3}H/^{14}C$, of same experiment. Maximum near *top* of gradient is due to cell debris.

The broad minimum in the isotope ratio curve at very large cells (11 mm/hr) could possibly be due to an inhibitory action of GIF on very early cells (CFU–C) of the granulopoietic system. These cells seem to be rather large (*13*). An effect of GIF on the unipotent committed stem cells would be compatible with the findings of Metcalf (*14*) which demonstrate that colony formation in agar by bone marrow cells is inhibited by a dialyzable substance(s) from bone marrow-conditioned medium. It is very probable that this substance, the molecular weight of which is less than 10,000 Daltons, may be identical with GIF.

The results obtained therefore indicate that

GIF inhibits the proliferation of all mitotically competent myeloid precursor cells of the bone marrow. Some points, however, of this hypothesis (CFU–C and myelocytes) require further investigation. It seems, nevertheless, that a single substance which is produced by the mature cells of the granulocytic series (*6*) inhibits the mitotic activity in the entire myeloid proliferation compartment and possibly even in the committed stem cell compartment. This would further imply that the responsiveness to the regulatory influences of GIF is already expressed at a very early stage of granulocytic differentiation. It may thus be that the presence of receptor sites for GIF is one of the fundamental properties of mitotically competent myeloid precursor cells, the expression of these sites being one of the early events of myeloid differentiation.

The results obtained may be summarized in the following way. GIF is produced by the mature cells of the granulocytic series and acts by inhibition of the proliferative activity of the myeloid precursor cells in the bone marrow. The action of GIF is strongly cell-type-specific—erythropoiesis is not inhibited (*5*). GIF shows no species specificity (*6*) as far as its biological action is concerned. The question may thus be raised as to whether GIF and the granulocytic chalone are the same substance.

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Commentary on "Chalone of the Granulocyte System," by T. Rytömaa, and "Granulopoiesis-Inhibiting Factor," by W. R. Paukovits [1]

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THESE TWO very interesting communications indicate a new family of controlling substances, and that we are dealing with some different kind of compounds than those which appear to be of the lymphocytic chalone family. The fact that there is a compound which inhibits the DNA synthesis in the bone marrow cells in vitro seems to be quite clear. The question really is: Is this compound, like the lymphocytic and epidermal material, a G_1 inhibitor (preventing cells moving from the G_1 into the S phase) or is it different from the other chalones insofar as it inhibits even cells which are already in the DNA synthetic period? Also, how specific is it for the granulocytic series of cells?

Now, I feel that the techniques used so far (i.e., short-term bone marrow incubations) are not really sensitive enough to give an answer to all these questions. In fact, some of the answers that we do have raise some suspicions in one's mind whether in fact the compound is S specific, as claimed. Let me reiterate this. In bone marrow, of the cells capable of DNA synthesis at any time, about half are granulocytic and the other half are erythrocytic. Consequently, if you incubate normal bone marrow and measure the DNA synthesis, then even if you can inhibit completely all those cells of say the granulocytic series which could have entered into S during a 4- to 5-hour incubation period, you would not expect a depression in DNA specific activity (or total activity) more than 10–20% at the most. If you are dealing with a polycythemic marrow in which you excluded all the erythroblasts, you will have improved the situation, but it would be extremely unlikely to expect, within a 4–hour incubation period, more than possibly 30–40% depression in total radioactivity, because that would be the maximal number of new cells recruited into the S period. In other words, as soon as you have depression of DNA synthesis exceeding 20–30% you will suspect that the compound is acting not only on the G_1–S transition but also on the S period. Furthermore, if in a normal marrow that contains both erythroid and granulocytic series, you are getting a depression exceeding 50% you must have inhibited completely not only all DNA synthesis in all S cells in the granulocytic series but also much of it in the erythroid series.

Now, in most cases, Dr. Rytömaa published, I believe, depressions ranging from 25 to 40%. We have repeated some of his experiments and in our system we are getting something like 50–60% depression. Admittedly, it is only one experiment, but this very large depression would, on the face of it, exclude the possibility that the only effect is on the granulocytic series of cells. Of course, one could say that the best evidence is the autoradiographic evidence, in which you are looking at the cells and seeing whether the normoblast labeling index is changed or the granulocytic grain counts are changed. Anyone who has worked with small-rodent bone-marrow autoradiography knows that such investigations require a degree of masochism because of the obvious uncertainties and imperfections of even the best preparations for this kind of quantitative analysis.

[1] Presented at the First Symposium of the International Chalone Conference, Brook Lodge, Augusta, Mich., June 5–7, 1972.

I put it to you, however, that there are techniques which could settle the problem more simply and in a much more quantitative fashion. One could look at the actual colony growth in vivo or the colony growth for the granulocytic series in vitro. You can use either normal mice or anemic mice, which you graft with bone marrow which will grow beautiful erythroid colonies in the spleen. The size of these colonies and the number of cells in them can be quantitatively measured, and the growth rate is measurable and predictable. This has been done many times in many laboratories. You can use polycythemic mice, which will never grow erythroid colonies because there is no erythroid demand in them and, therefore, all the splenic colonies will be granulocytic. Again, the population growth in these colonies can be counted. And, of course, you can grow the granulocytic colonies in vitro, where, again, the colony size as well as the number of colonies can be measured.

If we find, as I suspect we will, that the granulocytic colony growth is inhibited, the crucial question will be: Will the erythrocytic colony growth be inhibited as well? The techniques are available. They may require larger amounts of chalones but, until such experiments have been done, and while DNA depressions greater than 30–40% are found in short-term in vitro cultures, one is faced with the unhappy feeling that we are not quite certain about the specificity of the compounds.

MISCELLANEOUS CHALONES

Control of Fibroblast Proliferation [1, 2]

John C. Houck, Richard F. Cheng, *and* Vishwa K. Sharma,[3] *Biochemistry Research Laboratory, Children's Hospital of the District of Columbia, Washington, D.C. 20009, and Department of Child Health and Development, George Washington University Medical Center, Washington, D.C. 20037*

SUMMARY—The first portion of this paper describes the isolation and purification of a factor from serum which permits the mitosis of diploid human fibroblasts in culture and hence presumably is the mitogenic "turning on signal" which operates during the repair of a wound. The second portion addresses the question of the factor capable of inhibiting the mitosis of fibroblasts, the fibroblast "chalone." The relationship between the stimulating factor and the fibroblast chalone remains to be determined. Our preliminary information suggests that the serum factor displaces the chalone from the fibroblast surface.—Natl Cancer Inst Monogr 38: 161–170, 1973.

THE FIBROBLAST'S response to injury is to be turned on mitotically and subsequently to be turned off. The initial events of wound repair involve, besides epithelial proliferation and capillary proliferation, the proliferation of the fibroblast until these cells reach a high population density within the interstices of the wound space. They then cease to be mitotically active but become active biochemically in terms of synthesis of collagen and muccopolysaccharide. The question is: What makes the fibroblast proliferate and what makes it stop proliferating?

FIBROBLAST MITOGENESIS

For a number of years, it has been known that serum is an important supplement of chemically defined media for the cultivation of mammalian cell lines in vitro (*1–10*). Although certain established cell lines could be grown in specially supplemented, but serum-free, chemically defined media, these cell lines were heteroploid or myxoploid rather than diploid (*11–14*). Attempts to isolate from serum the essential component(s) for promoting the growth of mammalian cell lines in vitro have been made in several laboratories; however, the results appear to be contradictory (*2–10, 15, 16*).

We have found that diploid human embryonic lung-derived (WI–38) fibroblasts (*17, 18*) and fibroblasts derived from the biopsy of human skin stringently require serum for their multiplication (*18, 19*). Using Diaflo ultrafiltration membrane units, iso-electric focusing, and preparative gel electrophoresis, we have isolated the essential component from mammalian serum for the promotion of growth of diploid human fibroblasts in culture. This component could not be replaced by insulin, serotonin, trypsin, ACTH, growth hormone, gonadotropin, thyroxine, cortisol, or even urine (*3, 6, 9, 20, 21*).

[1] Presented at the First Symposium of the International Chalone Conference, Brook Lodge, Augusta, Mich., June 5–7, 1972.

[2] Supported by contract DADA 17–69–C–9142 from the US Army Medical Research and Development Command.

[3] We thank Mr. Richard Weil for his valuable technical assistance.

Two cell lines—WI-38 and GWF (adult human cutaneous biopsy)—were used in the course of this study. The karyotype of these cells was carefully monitored throughout the entire course of this study. These cells were used for no more than 35 doublings (WI-38) or 15 doublings (GWF) (*22*). Contamination of the cell lines by PPLO was excluded first on the basis of culture methods (*23*) and second on the basis of the autoradiographic demonstration that these cells did not incorporate thymidine-^{3}H into their cytoplasm but only into their nucleus.

The cells were normally cultivated in Eagle's minimal essential medium (MEM) supplemented with glutamine, streptomycin, penicillin, and 10% calf serum (10% GMS) in Leighton tubes. Approximately 4×10^4 cells were seeded into each Leighton tube. Experimental medium was made by adding various concentrations of the appropriate serum fractions to the MEM. After incubation in normal medium (10% GMS) overnight, the cultures were rinsed repeatedly with several portions of Earle's balanced salt solution (BSS) to remove both the serum and the cells that had failed to adhere to the glass surface. The experimental medium was then added to replicate fibroblast cultures and thereafter changed three times weekly. The numbers of cells/cm^2 were counted daily by using an inverted microscope and a ruled eyepiece as described previously (*17*). A log plot of the cell number versus incubation time was prepared and the slope of the curve during the logarithmic growth phase was determined. The rate of growth was expressed as "population generation time" in hours (*17*).

Effects of Serum Concentration

The generation times for both WI-38 and GWF fibroblasts were determined with various concentrations of serum (table 1). At low concentrations of calf serum there was a linear relationship between the rate of cell growth and the serum concentration in the medium. However, above 30% serum, an apparent toxicity of serum for these fibroblasts was found. It appeared that the optimal serum concentration in chemically defined medium was between 10 and 20%. Therefore, all control cultures were maintained in 10% serum.

A WI-38 cell line which had been transformed by incubation with simian virus 40 (SV40) (10^6 units/ml for 10 doublings) was also used. The SV40-transformed WI-38 cells were karyologically heteroploid. Viral transformation of these cells was also indicated by the loss of contact inhibition (*18*). These transformed cells did not require serum in the medium in order for them to divide. However, the addition of serum to the culture medium of these cells did decrease their generation time.

Molecular Filtration

Calf serum was subjected to ammonium sul-

TABLE 1.—*Effect of concentration of calf serum on generation time of human fibroblasts*

Serum concentration (vol %)	Serum protein (mg/ml)	Generation time, mean ± SD (hr)		
		WI-38*	GWF†	SV-WI-38‡
0.0	0.0	∞	∞	135±4.1
0.625	0.375	157±2.4	161±5.6	94±1.7
1.25	0.75	120±1.7	136±2.7	104±1.1
2.5	1.5	78±2.1	77±1.4	72±4.9
5.0	3.0	56±1.9	61±0.8	78±6.6
7.5	4.5	47±1.6	49±2.1	52±0.7
10.0	6.0	33±1.6	34±1.7	64±3.7
20.0	12.0	36±1.6	38±1.1	—
30.0	18.0	118±0.7	114±3.2	—
40.0	24.0	Detached	Detached	—
50.0	30.0	Detached	Detached	—

*Normal diploid fibroblasts derived from human embryonic lung.
†Normal diploid fibroblasts derived from human skin.
‡WI-38 transformed by cultivation with simian virus 40(10^6 units/ml) for 10 doublings.

TABLE 2.—*Growth-promoting activity of ultrafiltration fraction II on diploid human fibroblasts*

Medium	Generation time, mean ± SD (hr)	
	WI-38	GWF
GM + whole calf serum		
0 vol %; 0 mg/ml	∞	∞
10 vol %; 6 mg/ml	34±3.1	33±5.7
GM + UF II* (mg/ml)		
12.0	26±3.4	26±1.9
6.0	26±2.1	27±0.8
3.0	30±1.9	29±4.7
2.0	34±3.0	33±5.0
1.5	48±2.7	41±6.3
1.0	47±0.5	41±3.1
0.5	91±0.9	98±6.7

*Ultrafiltration fraction II.

fate precipitation in the usual manner in the cold. Five fractions resulted, all of which had an equal amount of growth-promoting activity toward diploid fibroblasts. Dialyzed lyophilized calf serum was reconstituted in 0.15M NaCl and subjected to Diaflo ultrafiltration membrane sieving [Amicon Corp., Mass. (*24*)] to separate it into four fractions according to molecular weight (>300,000, 300,000–100,000, 100,000–50,000, and 50,000–30,000 Daltons). The mitogenically active component was found exclusively in the fraction which contained serum proteins with molecular weights ranging from 50,000 to 100,000 Daltons (ultrafiltration fraction II). At concentrations of ultrafiltration fraction II of 2.0 mg/ml, the rate of growth was comparable to that with 10% serum (table 2). High concentrations of ultrafiltration fraction II did not inhibit fibroblast mitosis.

The rate of passage of this serum mitogenic activity through the 100,000–Dalton filter (Diaflo XM 100 A) was extremely slow. Thus it was assumed that the molecular weight of the component might be very close to 100,000 Daltons. For preparative purposes, therefore, we collected the serum fraction between 300,000 and 50,000 Daltons (fraction II).

Iso-Electric Focusing

Ultrafiltration fraction II was subjected to isoelectric focusing (*25*) in the LKB 8100 "Ampholine" electrofocusing device over a pH gradient from 4 to 6. The active fraction was found in fraction IEP-3 with an iso-electric point of pH 5.2–5.4 (text-fig. 1). The chemically defined medium supplemented with fraction IEP-3 at 0.125 μg/ml gave population doubling times comparable to those found for WI-38 fibroblasts in medium supplemented with 10% calf serum (table 3). Again, no cytotoxic effect against WI-38 fibroblasts was observed with high concentrations of this fraction.

Preparative Electrophoresis

Fraction IEF-3 was subjected to preparative gel electrophoresis in a refrigerated Buchler Polyprep-200 (Buchler Instrumentation Division, Fort Lee, N.J.) at pH 9.5 and a current of 50 mA. The mitogenically active fraction was found in pool 2 (fraction PE-2) (text-fig. 2.) As shown in table 4, chemically defined medium supplemented with fraction PE-2 at 50 μg/ml gave population doubling times similar to those found for WI-38 fibroblasts in medium supplemented with 10% calf serum. Tests with combinations of fractions indicated that only PE-2 contained fibroblast mitogenic activity.

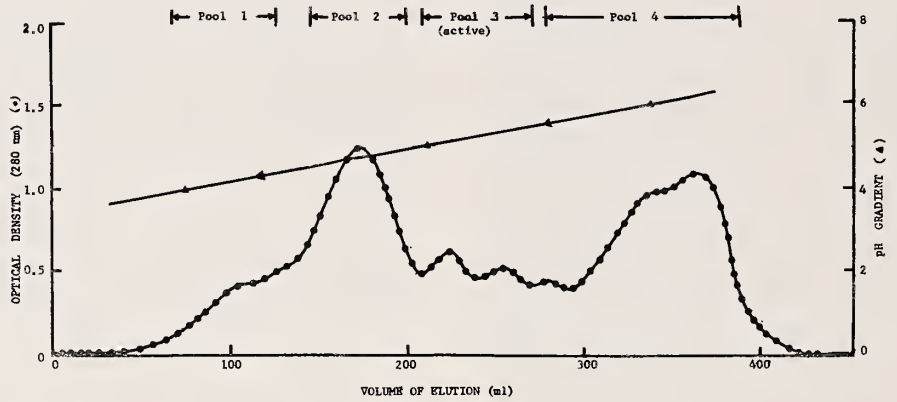
Molecular Homogeneity and Properties

On acrylamide gel electrophoresis, fraction IEF-3 was essentially composed of three different

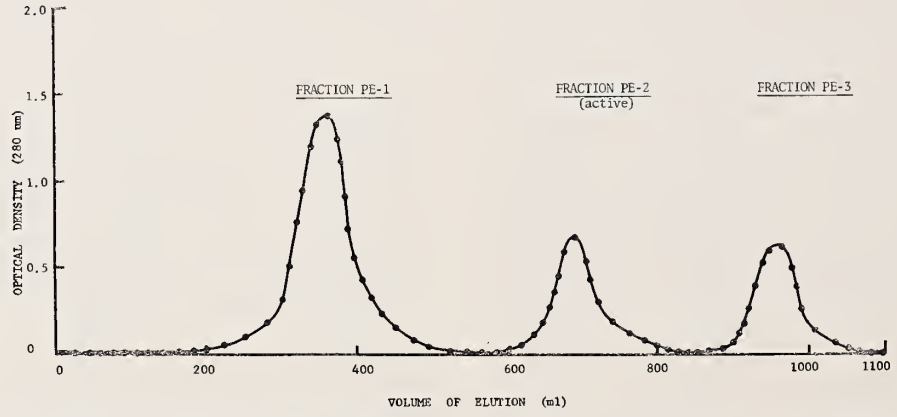
TABLE 3.—*Growth-promoting activity of fraction IEF-3* on diploid human fibroblasts*

Medium	Generation time, mean ± SD (hr)
	WI-38
GM + whole calf serum	
0 vol %; 0 mg/ml	∞
10 vol %; 6 mg/ml	34±2.4
GM + fraction IEF-3* (mg/ml)	
2.0	29±3.4
1.0	27±1.9
0.5	28±4.1
0.25	30±1.5
0.125	32±4.9
0.100	45±1.7
0.050	51±0.7
0.025	119±10.6

*The active fraction isolated during iso-electric focusing of ultrafiltration fraction II.



TEXT-FIGURE 1.—Iso-electric focusing, with LKB 8100 "Ampholine" electrofocusing device, of proteins and fibroblast mitogenic activity of ultrafiltration fraction II from calf serum.



TEXT-FIGURE 2.—Preparative acrylamide gel electrophoresis, with Buchler "Polyprep 200" refrigerated apparatus at 50 mA and pH 9.5, of mitogenically active pool 3 from iso-electric focused and sieved calf serum.

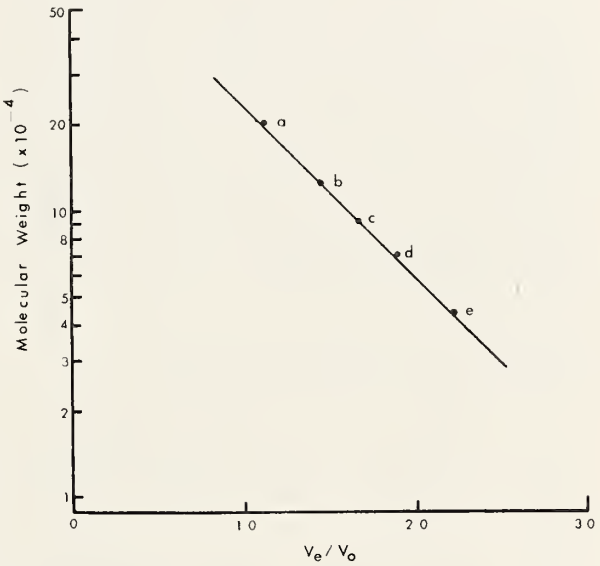
TABLE 4.—*Growth-promoting activity of fractions isolated by preparative gel electrophoresis*

Protein (mg/ml)	Generation time, mean ± SD (hr)		
	Fraction PE-1	Fraction PE-2	Fraction PE-3
0.250	179±16.8	29±4.3	∞
0.125	∞	27±3.8	∞
0.100	∞	30±2.7	∞
0.050	∞	34±5.4	∞
0.025	∞	57±7.1	∞
0.0125	∞	116±9.5	∞
		Combination of fractions	
PE-1 and PE-2, 0.050 mg/ml each		37±3.7	
PE-1 and PE-3, 0.050 mg/ml each		∞	
PE-2 and PE-3, 0.050 mg/ml each		40±5.1	
PE-1, PE-2, and PE-3, 0.025 mg/ml each		58±7.5	

components. After preparative gel electrophoresis, fraction PE-2 contained only one electrophoretic band on analytical gel electrophoresis (fig. 1). On gel electrophoresis at three different pH values, only one electrophoretic band was revealed in fraction PE-2 (fig. 2). By comparing the electrophoretic pattern of fraction PE-2 with that of standard serum proteins, it appeared that the mitogenically active component of serum moved as an electrophoretically homogeneous β-globulin.

Molecular Size

For determination of molecular weight, 8 mg of the serum-derived mitogen for diploid human fibroblasts in 3 ml of 0.05M acetate buffer (pH 5.5) was run through a Bio-Gel P-200 column (2.5 by 100 cm) (*26*). The results indicate that the fibroblast mitogen is about 120,000 Daltons (text-fig. 3).



TEXT-FIGURE 3.—Estimation of molecular weight (in Daltons) of 8 mg of purified fibroblast mitogenic factor from calf serum in 3 ml of 0.05M acetate buffer (pH 5.5) by elution ratios from 100 by 2.5 cm column of Bio-Gel P-200 column. *a*) Collagen (β-chain) (gelatin); *b*) mitogenic factor; *c*) transferrin; *d*) albumin; *e*) ovalbumin.

General Properties

This serum-derived component responsible for promoting the growth of diploid human fibroblasts appears to be thermostable at 56° C for 30 minutes. It is inactivated by both trypsin and neuraminidase. On thiobarbituric acid assay for sialic acid (*27*), it was found to contain 1.2% sialic acid. The total carbohydrate content was 3.4% by the anthrone reaction (*28*).

By using a variety of media such as NCTC, CMRL, and medium 199, we attempted repeatedly to substitute for either serum or purified serum factor in terms of stimulating the mitosis of diploid human fibroblasts. We were unable to do so. However, we were able to cultivate both WI-38 and cutaneous derived fibro-

blasts for 12 doublings by using the purified factor in MEM at 50 μg/ml.

Similar thermostable fibroblast mitogenic material, having a molecular weight of 120,000 Daltons and an iso-electric point of pH 5.2 to 5.4, was isolated from the sera of fetal calf, cow, horse, and human. Each material represented approximately 0.5–0.7% of the proteins in the whole serum.

Ultrafiltration through Diaflo molecular membranes gave a purification of about threefold, iso-electric focusing gave another purification of 22-fold, and, after preparative acrylamide gel electrophoresis, these homogeneous mitogenic products had been purified about 125-fold from the circulating concentration.

It is interesting that this serum mitogenic factor for fibroblasts was markedly inactivated (presumably by denaturation) during both DEAE- and CM-cellulose column chromatography and during preparative acrylamide gel electrophoresis at a current of 150 mA. In the latter circumstance, all of the mitogenic activity was lost despite its thermostability and the excellent refrigeration system of the Buchler preparative gel apparatus!

Comparison With Other Serum Factors

The properties of this circulating mitogen for diploid human fibroblasts differ markedly from those of mitogenic materials obtained previously from serum, such as fetuin (*7*) (40,000 MW; iso-electric pH 3.4); the α-1 protein of Holmes (*4*), and the thermolabile materials of Holley and Kiernan (*6*) and Lieberman and Ove (*3*). It is too large to be related to the various proteins reported by Paul et al. (*15*) to be mitogenic for 3T3 mouse fibroblasts (which, despite being "contact inhibited," we have found to be heteroploid). The growth-promoting serum proteins described by Michl and Rezacova (*16*) were α_1-globulins, and the two serum protein fractions reported by Healy and Parker (*10*) were 40,000–50,000 and 850,000 Daltons and were neuraminidase stable. Only the thermostable serum factor of 100,000–150,000 Daltons described by Todaro et al. (*5*) would seem to be similar to our mitogenic factor.

THE FIBROBLAST CHALONE

Approximately 2×10^5 WI-38 cells are introduced into small Falcon flasks (25 cm^2 area) in 10% serum and MEM as described above. Twenty-four hours later, the supernatant medium is decanted, the cells which adhere to the surface of the plastic are rinsed with serum-free medium, and then new medium containing 10% calf serum is supplied to the cells at 2 ml/culture. The cells are then allowed to incubate for 24 hours with 1 μCi of ^{3}H-labeled thymidine. After this period, the medium is removed; the cells are rinsed with serum-free MEM twice, trypsinized, harvested, counted in a hemocytometer, and then mixed with 5% cold trichloroacetic acid (TCA). The resulting precipitate is rinsed once again with TCA in the cold and allowed to stand in suspension overnight with 5 ml of TCA. On the next morning, the suspension is centrifuged, the supernatant is removed and three more rinsings with 5% TCA are accomplished to remove all of the nonspecifically adsorbed thymidine-^{3}H from the precipitated RNA, DNA, and protein. After this rinsing, the final precipitate is solubilized in NCS solubilizer at 80° C (0.5 ml). This solubilized material is then mixed with a liquid scintillation "cocktail" and counted in a liquid scintillation counter in the usual fashion. The results are expressed as cpm/10^6 cells.

Diploid human fibroblasts were grown to a confluent monolayer in large bottles. These cells were rinsed thoroughly with isotonic saline three times to remove adhering serum and other materials and then were collected by trypsinization and sonically disrupted in isotonic saline. After centrifugation of the sonically disrupted cells in the cold, the clear supernatant was removed and dialyzed against 200 volumes of water in the cold. After dialysis, the contents of the dialysis bag were centrifuged and the clear supernatant was lyophilized.

The inhibitory effect of various concentrations of these fibroblast extracts on the incorporation of thymidine-^{3}H by diploid human fibroblasts in culture as described above is demonstrated in table 5. At a concentration as low as 500 μg/ml,

TABLE 5.—*Inhibitory effects of fibroblast extract concentration on incorporation of thymidine-^{3}H by diploid human fibroblasts*

Extract (mg/ml)	Thymidine-^{3}H uptake (cpm/10^6 cells)	Inhibition (%)
0	3,118	—
0.250	2,253	28
0.500	833	73
1.0	889	71
2.0	785	75

fibroblast extract will inhibit 73% of the control uptake of thymidine-^{3}H by these cells.

This particular extract at a concentration of 1 mg/ml had no effect upon the thymidine-^{3}H uptake by diploid human lymphocytes when stimulated by phytohemagglutinin (PHA) in culture and similarly had no effect on the thymidine-^{3}H uptake by HeLa cells in culture.

The kinetics of the inhibitory effect of this dialyzed and lyophilized extract from diploid human fibroblasts on the uptake of thymidine-^{3}H by diploid human fibroblasts in culture in log phase was explored, with the fibroblast extract at 330 μg/ml. Within 3 hours there was a significant effect on the uptake of DNA precursor by fibroblasts exposed to the fibroblast extract (table 6).

Two doses of extract (0.5 and 2.0 mg/ml) were added to the incubation medium of diploid human fibroblasts in culture. Half of these cultures were then rinsed with MEM containing 10% serum twice, and then new medium, supplemented with 10% serum, was added along with thymidine-^{3}H. Most of the inhibitory activity of the fibroblast extract at 0.5 mg/ml was removed by the rinsing (table 7). At an extract concentration of 2 mg/ml, only two-thirds of the inhibitory activity was removed by washing the cells. This indicates that the bulk of the mitotic inhibition effected by fibroblast extract was reversible when these cells were rinsed with MEM containing 10% serum, but at higher concentrations of inhibitor not all of this inhibition is reversible. However, these results suggest that cytotoxicity is probably not a very large issue. Morphologic studies suggest that there is no cytotoxicity in these extracts.

The extract of diploid human fibroblasts was subjected to molecular sieving using Amicon-Diaflo filters. From the morphologic appearance and the cell numbers, almost all of the mitotic inhibitory activity of the fibroblast extract that could be demonstrated upon diploid human fibroblast in log phase growth was found in the fraction between 30,000 and 50,000 Daltons. This molecular weight is essentially similar to that proposed for the epidermal chalone and described by us for the lymphocyte chalone (this conference).

TABLE 6.—*Kinetics of thymidine-^{3}H uptake by diploid human fibroblasts in presence and absence of fibroblast extract (330 μg/ml)*

Hours	Thymidine-^{3}H uptake (cpm/10^6 cells)		Inhibition (%)
	Control	Inhibitor	
2	277	227	18
3	460	267	42
4	807	407	50
24	4,266	2,026	53

TABLE 7.—*Effect of rinsing inhibitor-treated fibroblasts on subsequent incorporation of thymidine-^{3}H*

	Control	Inhibitor at 0.5 mg/ml		Inhibitor at 2.0 mg/ml	
		No rinse	After rinse	No rinse	After rinse
Thymidine-^{3}H (cpm/10^6 cells)	4,995	1,135	4,475	1,205	3,614
	4,868	1,240	4,727	1,234	3,834
Mean	4,931	1,187	4,601	1,219	3,724
Inhibition (%)	—	76	7	75	24

Diploid human fibroblasts in culture were maintained in confluency for 3–4 days in MEM containing 10% serum and supplemented as described above. At the end of this time, the medium was removed, dialyzed exhaustively against water, and centrifuged; the clear supernatant was lyophilized. The effect of the nondialyzable portion of this used medium at various concentrations, on the thymidine-^{3}H uptake by diploid human fibroblasts in log phase growth, was explored in the manner described above. In this series of experiments, the quantitative uptake of thymidine-^{3}H was approximately 3,200 cpm/10^6 cells, and up to 77% of this uptake could be inhibited by the addition of significant amounts of the nondialyzable portion of used medium from other diploid human fibroblasts (table 8). There was no nondialyzable mitotic inhibitor in either normal MEM supplemented with 10% serum but not exposed to cells or similar medium which had been exposed to cells for only 24 hours. Apparently, equivalent amounts of inhibitory mitotic activity could be demonstrated after either 3 or 4 days of incubation with diploid human fibroblasts in culture.

This inhibitory activity of used medium was not associated with any apparent cytotoxicity, as judged by morphologic criteria in culture.

The dialyzed and lyophilized used medium was then subjected to iso-electric focusing as described above; the components were resolved into three major peaks with iso-electric points of 3.5–4.3, 4.4–4.9, and 5.0–5.7. At 330 μg/ml, only the first peak contained significant amounts of inhibitory activity against fibroblast mitosis (table 9). The apparent inhibition, of 16%, by peak III is not statistically significant.

When the peak I material was subjected to molecular sieving, the mitotic inhibitory activity was only in the molecular weight range of 30,000–50,000 Daltons. As little as 50 μg/ml of this fraction could inhibit 50% of the thymidine-^{3}H uptake by diploid human fibroblasts in log phase growth in culture.

These data suggest to us that extracts of fibroblast in culture and the nondialyzable portion of the "used medium" from these cells contain a mitotic inhibitor which is not cytotoxic for fibroblasts and which apparently is specific for these fibroblasts. The molecular weight of this material is between 30,000 and 50,000 Daltons and its iso-electric point is between 3.5 and 4.3.

This mitotic inhibitory activity was found to be destroyed by trypsin digestion and to be thermolabile (50° C for 30 min).

These general properties of this mitotic inhibitory activity are similar to those described for the epidermal chalone and the lymphocyte chalone, leading us to believe that fibroblast mitosis might also be under chalone control.

TABLE 8.—*Effect of nondialyzable portion of used medium on thymidine-^{3}H uptake by diploid human fibroblasts*

		Dose		
	Control	2.0 mg/ml	1.0 mg/ml	0.5 mg/ml
Thymidine-^{3}H uptake (cpm/10^6 cells)	3,221	824	1,160	2,343
	3,209	684	1,311	2,583
Mean	3,210	754	1,235	2,463
Inhibition (%)	—	77	62	23

TABLE 9.—*Effect of three fractions from iso-electric focusing of nondialyzable portion of used medium on fibroblast uptake of thymidine-^{3}H*

		Fractions, at 330 μg/ml		
	Control	Peak I pH 3.5–4.3	Peak II pH 4.4–4.9	Peak III pH 5.0–5.7
Thymidine-^{3}H uptake (cpm/10^6 cells)	4,152	2,332	5,012	3,892
	4,292	2,020	5,046	3,186
Mean	4,222	2,176	5,029	3,539
Inhibition (%)		50	0	16

The relationship between the serum mitogenic factor and the fibroblast chalone is not clear. Preliminary evidence suggests that the mitogenic factor from serum displaces the chalone from its binding site on the cell; as a consequence of displacing this chalone into the medium, the fibroblast then enters S phase. For this reason, a significant amount of fibroblast chalone can be recovered from incubation medium containing 10% serum. The mechanism of action of both the mitogenic factor and the chalone and the relationship of one to the other need to be determined.

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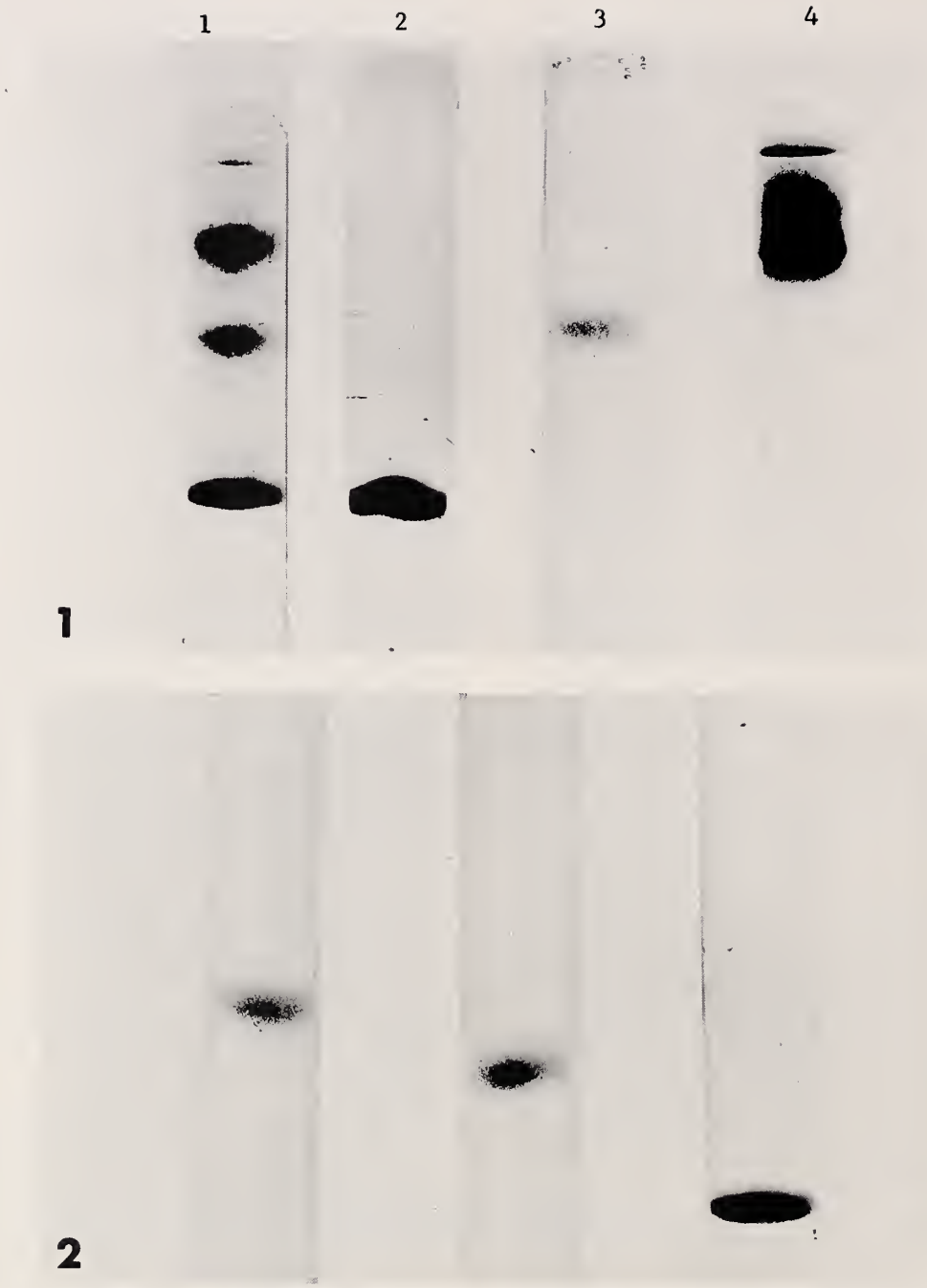

FIGURE 1.—Analytical acrylamide gel electrophoretic patterns of 150 μg samples of preparative electrophoretic fractions of electrofocused mitogenic fraction from calf serum. 1) Iso-electric focused pool 3 (pH 5.2–5.4). 2) Electrophoretic fraction PE-1 (inactive). 3) Electrophoretic fraction PE-2 (active). 4) Electrophoretic fraction PE-3 (inactive).

FIGURE 2.—Acrylamide gel electrophoresis of 150 μg fraction PE-2 at different pH values. *Left,* pH 9.5. *Center,* pH 8.0. *Right,* pH 4.3 (polarity reversed).

Commentary on "Control of Fibroblast Proliferation," by J. C. Houck, R. F. Cheng, and V. K. Sharma[1]

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THE PAPER by Houck, Cheng, and Sharma deals with two factors having distinctly different biological activities. One promotes diploid fibroblast proliferation in vitro. The other decreases the amount of radioactivity in WI-38 cells in vitro after exposure to tritiated thymidine. Neither activity was expressed in cultures of two other types of human cells under comparable conditions. This indicates that the factors responsible for the biological activities were specific in their actions on human diploid fibroblasts. Such specificity of biological activity is of fundamental importance to the concept of chalones and their role in physiology. Also, the differential effect against several human cell types indicates that the biological effect was not due to nonspecific toxicity of the preparations.

The suggestion that the substances which caused the expression of these two activities may be intimately related in a competitive manner is an intriguing possibility. It can be consistent with some of the thoughts expressed by Mueller at the outset of this conference regarding receptor sites on cell plasma membranes and with the capacitor concept.

Over the past two decades the isolation and successful application in vitro of a mitogenic factor from serum have always been exciting to any cell or tissue culturist. There are a number of people in laboratories around the world who are "taking serum apart," just as Houck and his associates have done [e.g., (*1–4*)]. Much of this work on serum fractionation stems from the so-called phenomenon of contact inhibition of growth in vitro. That is, when diploid cells reach confluency in vitro, they usually but not always cease to divide. However, if one impinges fresh serum onto the confluent sheet of cells, some of the cells resume DNA synthesis, and, if this is done repeatedly as in perfusion culture, the population of cells becomes multilayered (*5*). Thereupon, if one pulses the culture with tritiated thymidine, labeled nuclei are found in the top, in the middle, and in the bottom layers, as we reported several years ago (*6*).

Human diploid fibroblasts have a finite lifespan in vitro—i.e., the ones derived from embryonic tissue usually die out in culture after about 40–50 population doublings. Cells set up in culture from adult human tissues die out sooner (*7*). Each doubling is usually referred to as a passage. On the other hand, under identical conditions, cancer cells and heteroploid cells are immortal, so to speak, in culture. During the senescence phenomenon of human diploid cell populations in vitro, with increasing passage number there is a decreasing percentage of cells capable of undergoing mitosis (*8,9*).

In regard to Houck and associates' description of the two biological activities expressed by their isolated materials, a number of possibilities are obvious. Will the lifespan of WI-38 cells be "normal" in vitro when continuously subcultured in nutrient medium containing the mitogenic factor? Can WI-38 or other human diploid fibroblast cultures multilayer when challenged with the mitogenic factor? With respect to the 30,000–50,000 Daltons material obtained from extracts of WI-38 monolayers, it will be most interesting to see if this material affects in

[1] Presented at the First Symposium of the International Chalone Conference, Brook Lodge, Augusta, Mich., June 5–7, 1972.

[2] Dr. Kruse died on Jan. 31, 1973.

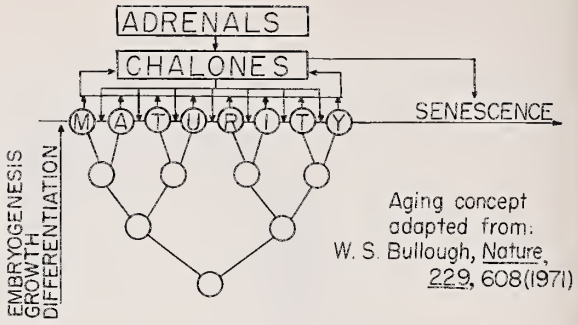
any way the established and reproducible fact that the percentage of cells incapable of labeling their nuclei with tritiated thymidine increases with the age of the cells in vitro. Among other things, this could be relevant to Bullough's thesis of the role of chalones in aging (*10*). Conceivably, also, it is of fundamental importance to the question of whether the finite lifespan in vitro has relevance to such occurrences in vivo.

Houck asked me to talk about perfusion culture systems, their applications, and their relevance to the underlying subject of this conference—homeostatic mechanisms. For about 10 years we have cultured a variety of cells in perfusion systems, adaptable both to stationary vessels, such as T-flasks, and to roller bottles. These systems and their application to various aspects of biological inquiries have been described elsewhere (*11*).

These perfusion systems were primarily designed for working with cells in vitro under environments approaching steady-state conditions, to study nutrition and metabolism. The fact that one usually obtains greatly enhanced cell yields with them was of secondary consequence. All living systems are open systems. Therefore, it is surprising to consider that almost all work with mammalian cells in vitro, including assay of chalones, has been done in closed culture systems whose environments are in a constant state of flux.

Among other things, what one obtains in perfusion systems is a relatively constant pH, constant levels of nutrient input, and constant levels of metabolic product concentrations. These are three variables that it is often desirable to eliminate in working with animal cells in vitro, whether one is studying mitotic or nonmitotic cell populations. If we can replace the whole serum complement in the influent stream with the mitogenic factor described by Houck and associates, it may be of much significance in many applications, including the study of chalones.

What is the reason or reasons why human diploid fibroblasts die out in culture, and might chalones be implicated in any way in this process? Text-figure 1 is an adaptation of a sketch



from one of Bullough's papers in *Nature* last year (*10*).

Bullough suggests that chalones affect the rate at which epidermal cells progress in the process of differentiation to keratinizing cells and ultimate death. I have already alluded to the fact that, as WI-38 populations grow older, their proportion of cells incapable of DNA synthesis, as measured by thymidine uptake, increases. An intriguing experimental plan would be to perfuse cells with the serum mitogenic factor, switch them into a maintenance state for long periods of time by removing the mitogenic factor, recirculate the "conditioned" culture medium, and determine not only the percentage of cells incapable of thymidine uptake but also the alteration, if any, of the total lifespan of human diploid cells in vitro.

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The Hepatic Chalone [1, 2]

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SUMMARY—We give the name "liver chalone" to the factor responsible for the inhibition of DNA synthesis in the regenerating liver; the tissue specificity which qualifies this factor for this name will be shown and the purification of the liver chalone and studies of its mode of action are described.—Natl Cancer Inst Monogr 38: 175–184, 1973.

LIVER REGENERATION (*1*)

The liver is formed of polygonal lobules surrounded by connective tissue containing branches of the portal vein and the hepatic artery. The lobule consists of hepatocytes forming trabeculae separated by sinusoid capillaries leading to a central vein; the blood flows from the periphery toward the center of the lobule. In adult liver, only a few hepatocytes, localized in the immediate vicinity of portal vessels, are still dividing. The cellular cycle has a G_1 of 9 hours, an S of 9 hours, and a G_2 + M of 3–4 hours. All the other hepatocytes in the lobule are stationary; they are blocked in G_0.

In the young-adult rat, about 15 hours after a subtotal hepatectomy that removes 70% of the liver mass, hepatocytes which were previously blocked in G_0 resume DNA synthesis—i.e., proceed into S. These hepatocytes are localized at the periphery of the lobule. The resumption of DNA synthesis in the hepatocytes propagates as a wave toward the center of the lobule. By 24 hours after the subtotal hepatectomy, 30% of the hepatocytes are in S; this percentage decreases quickly after this. The DNA synthesis wave is followed, after a delay of 4–5 hours, by a wave of mitoses, which also propagates from the periphery toward the center of the lobule.

Nearly all the hepatocytes are involved in regeneration. If, during the 7–8 days needed for the liver to recover to a normal mass, thymidine-^{3}H is given continuously, 95% of the hepatocytes are found to be labeled by autoradiography. During regeneration, most of the hepatocytes divide only once; a few do it twice or, at most, three times.

Numerous experiments show that humoral factors control liver regeneration. Some authors think it is a stimulatory factor whose activity is increased; other authors think that an inhibitory factor disappears. It is possible that both kinds of factors exist and that the growth of the hepatic tissue depends on a delicate equilibrium between stimulatory and inhibitory factors.

THE CHALONES

Several instances have been described of tissues containing and secreting a factor that inhibits DNA synthesis and mitosis in their own cells. These factors have been called "chalones" by Bullough (2) who studied particularly the epidermal chalone and the chalone of the

[1] Presented at the First Symposium of the International Chalone Conference, Brook Lodge, Augusta, Mich., June 5–7, 1972.

[2] This work was done in collaboration with Y. Deschamps, J. Pushpathadam, M. Desrosiers, and M. Boucher. It was supported by grants from the Canadian Medical Research Council and the National Cancer Institute of Canada.

melanocytes. Chalones are tissue-specific—e.g., the epidermal chalone acts only on the cells of the basal germinative layer of the epidermis; it has no effect on the sebaceous glands or on the melanocytes. On the other hand, the chalone is not species-specific—pig epidermal chalone is active on mouse epidermis.

The chalone quickly inhibits DNA synthesis. I shall discuss later whether the chalone only stops progression from the G_1 to the S phase of the cellular cycle or whether it is also able to stop DNA synthesis when the cell is already in the S phase. The chalone inhibits mitosis and this effect is also quite rapid, indicating that it cannot be the consequence of DNA synthesis inhibition but rather of a block at the G_2 to M transition. We do not know whether the same molecule acts on the S phase and on the G_2 to M transition.

Chalone may be only one of several factors controlling cell multiplication in a tissue. Rytömaa and Kiviniemi (*3*) have described a chalone which inhibits the multiplication of granulocytes, a granulopoietin which stimulates their multiplication, and an antichalone which neutralizes the action of the chalone. It thus seems that tissue growth might be dependent on a delicate balance between simulatory and inhibitory factors.

Does a chalone control the growth of the liver and also the liver regeneration after partial hepatectomy?

Several experiments can be interpreted as showing the existence in normal blood of a factor inhibiting liver growth. Stich and Florian (*4*) observed that an intraperitoneal injection of rat plasma to a subtotally hepatectomized rat inhibits mitosis in the liver remnant of the recipient animal when the plasma donor has an intact liver but not when it is also hepatectomized. When Goutier et al. (*5*) perfused a liver lobe taken a few hours after partial hepatectomy, the early nuclear RNA synthesis occurred if the blood used for the perfusion came from hepatectomized animals but not if the blood came from intact animals.

This humoral inhibitory factor might come from the liver. Indeed, already in 1956, Saetren (*6*) demonstrated that an intraperitoneal injection of a whole liver macerate decreases the mitotic index in the regenerating liver or the liver of young animals. The effect is quite specific for the hepatocytes; the nonparenchymal cells are not affected. The intraperitoneal injection of a kidney macerate had no such action.

We started with a similar approach, but, not being morphologists, we looked for the inhibition of DNA synthesis. We started with the following in vivo experiment. The liver of an adult rat was homogenized in saline (3 ml of 0.85% NaCl/1 g of liver); the homogenate was centrifuged at 105,000 × *g* for 100 minutes, and the supernatant fraction was decanted. Fifty-gram rats were partially hepatectomized at 10 AM; 21 hours later, they were given intraperitoneal injections of 2 ml of this liver supernatant or 2 ml of saline for control. At 4 hours later, they received an intraperitoneal injection of 100 μCi of thymidine-^{3}H. The remaining lobes were removed 1 hour later and the specific radioactivity of their DNA was determined (specific radioactivity expressed as dpm/1,000 × A_{600}; A_{600} = absorbance, with diphenylamine reagent, at 600 nm).

There was a significant decrease of the DNA specific radioactivity in the rats given 2 ml of liver supernatant (table 1).

TABLE 1.—*Inhibition of DNA synthesis in rat liver by rat liver supernatant*

Treatment	A_{600} × 1,000	dpm	dpm/1,000 A_{600}	Mean ± SD
2 ml supernatant	132	72,289	548	408±78
	138	54,859	397	
	157	43,852	279	
2 ml saline	137	144,942	1,058	829±115
	150	109,969	733	
	146	101,592	696	

ASSAY FOR LIVER CHALONE (7)

To purify a biological factor, one needs a quantitative assay method. The in vivo assay described above was too laborious and used too much chalone. Also, there was a great deal of variation from rat to rat, which obliged us to use several animals for the assay and several animals for the control. We thus started to devise an in vitro method in which the same liver could be used for the assay and the control. The following method was developed.

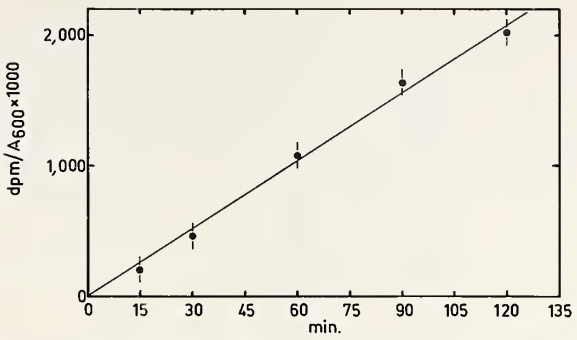
Seventy-gram male rats are subtotally hepatectomized (removal of 70% of the liver mass) at 9 AM; 24 hours later (at the time of the highest DNA synthesis), the remaining lobes are removed and cut into slices approximately 0.5 mm thick with a Stadie-Riggs microtome. Each slice is cut in two, one-half for the assay and the other half for the control. The slices are kept at 0° C in Hanks' solution in 25-ml Erlenmeyer flasks. Four flasks, forming two pairs, are prepared from each residual liver.

We first studied the DNA synthesis in such slices without adding a chalone preparation. To each flask were added 20 μCi of thymidine-^{3}H (6 Ci/mmole) and Hanks' solution up to 5 ml. The flasks were then put in a 37° C water bath and shaken gently. After 2 hours of incubation, the DNA specific radioactivities were measured.

The oxygenation of the slices is critical. DNA synthesis was fivefold higher in a 95% O_2–5% CO_2 atmosphere than in a 95% air–5% CO_2 atmosphere. Consequently, an O_2–CO_2 atmosphere was used throughout our work.

We have also shown that, in a control not containing chalone, the incorporation of radioactivity from the 20 μCi of thymidine-^{3}H added at time 0 of incubation into the DNA of the regenerating liver slices is linear for 2 hours (text-fig. 1).

We next turned to the study of the error in the DNA specific radioactivity measurement in the control. Because each slice is divided in two pieces which are shared between paired flasks, each residual liver provides for four flasks (two pairs). As shown in table 2, 11 rats were used; 2 were excluded because the DNA specific radioactivities in the slices of their regenerating liver



TEXT-FIGURE 1.—DNA specific radioactivities after various incubation times when 20 μCi of thymidine-^{3}H (6 Ci/mmole) but no chalone preparation was added at time 0 to each of five flasks containing regenerating liver slices in 5 ml of Hanks' solution.

were too far from the general mean, so that only 36 results were submitted to statistical analysis. The general variation coefficient—i.e., the standard deviation from flask to flask, whatever the origin of the slices it contained—was 16%, while the residual variation coefficient between flasks prepared from the same liver was 13%; the difference is due to the variation from rat to rat, which is lower than in the in vivo assay. The residual variation coefficient between flasks of the same pairs was only 7%, much smaller than the residual variation coefficient between flasks from the same liver (13%). We believe that this difference is due to the fact that, in spite of the use of a microtome, the slices are of unequal thicknesses and that oxygenation is a critical factor for DNA synthesis. Whatever the explanation, we decided always to use paired flasks for assay and control in further work; in these conditions, a difference of 10% between control and assay can be considered significant at the confidence level of the standard error.

DOSE-EFFECT RELATIONSHIP OF LIVER CHALONE ON DNA SYNTHESIS

Rabbit liver was used for the preparation of liver chalone. The liver was homogenized in water (3 ml of water/g of liver) and the homogenate was centrifuged at 105,000 × g for 100 minutes. This supernatant was the source of

TABLE 2.—*Statistical analysis* of DNA specific radioactivation after 2-hour incubation of rat liver slices with thymidine-^3H*

Rat (i)	Slice No. (ijk)	DNA specific radioactivities x_{ijk}	m_{ij}	m_i	m
1	111	1174			
	112	1265	1220		
	121	1131			
	122	1042	1087	1153	
2	211	1904			
	212	1768	1836		
	221	1592			
	222	1544	1568	1702	
3	311	1340			
	312	1240	1290		
	321	1690			
	322	1590	1640	1465	
4	411	1521			
	412	1569	1545		
	421	1329			
	422	1230	1280	1412	
5	511	1342			
	512	1482	1412		
	521	1255			
	522	1277	1266	1339	
6	611	1564			
	612	1565	1565		
	621	1927			
	622	2214	2071	1818	
7	711	1373			
	712	1289	1331		
	721	1502			
	722	1728	1615	1473	
8	811	1560			
	812	1195	1378		
	821	1741			
	822	1672	1707	1542	
9	911	1329			
	912	1230	1280		
	921	1450			
	922	1560	1505	1392	1477
10	1011	461			
	1012	571	516		
	1021	511			
	1022	422	467	491	
11	1111	3103			
	1112	3380	3242		
	1121	3680			
	1122	3798	3739	3490	

*General variation coefficient=

$$\sqrt{\frac{(x_{111}-m)^2 + \ldots\ldots + (x_{922}-m)^2}{35}} = 240, \text{ i.e., } 16\%.$$

Residual variation coefficient between slices from the same liver=

$$\sqrt{\frac{(x_{111}-m_1)^2 + \ldots\ldots + (x_{922}-m_9)^2}{27}} = 192, \text{ i.e., } 13\%.$$

Residual variation coefficient between slices from the same pair=

$$\sqrt{\frac{(x_{111}-m_{11})^2 + \ldots\ldots + (x_{922}-m_{92})^2}{18}} = 104, \text{ i.e., } 7\%.$$

liver chalone in our first study of the dose-effect relationship.

A fraction of the rabbit liver supernatant was added to the slices kept at 0° C in Hanks' solution already containing 20 μCi of thymidine-^3H, and the volume was brought to 5 ml with more Hanks' solution. The control flask received H_2O instead of the liver supernatant. The flasks were then incubated for 2 hours at 37° C. Table 3 gives the DNA specific radioactivity as a function of the amount of protein in the fraction of the rabbit liver supernatant. We have defined the inhibited fraction of thymidine-^3H incorporation in DNA (IF) as:

(IF) as: $$IF = \frac{DNA\ sp.rad.(control) - DNA\ sp.rad.(assay).}{DNA\ sp.rad.(control)}$$

Text-figure 2 shows that the inhibited fraction is related to the amount of chalone (A) in a very simple way:

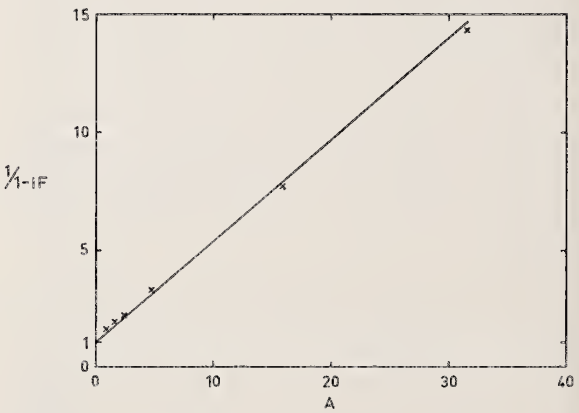
$$\frac{1}{1-IF} = 1 + kA. \qquad [1]$$

Such a relationship suggests the presence, in the hepatocytes, of receptor sites for the chalone molecules:

$$R + chal \rightleftarrows R - chal$$

$$K = \frac{(R-chal)}{(R)\ (chal)}$$

K = constant measuring the affinity of the receptor site for the chalone; (R) and $(R-chal)$ = numbers of receptor sites free or occupied by chalone molecules, respectively; and $(chal)$ = concentration of free chalone.



TEXT-FIGURE 2.—DNA synthesis inhibited fraction (IF) as function of amount, A (mg protein), of 105,000 × g supernatant of rabbit liver in 5 ml of incubation medium.

TABLE 3.—*Dose-effect relationship for liver chalone*

Protein (mg)	DNA specific radioactivity (dpm/1,000 A_{600})		IF	1/(1−IF)
	Control	Assay		
0.95	1,692	1,085	0.36	1.6
1.58	1,062	554	0.48	1.9
2.37	950	441	0.54	2.2
4.74	1,699	505	0.70	3.3
15.8	1,652	212	0.87	7.7
31.6	1,857	131	0.93	14.3

TABLE 4.—*Influence of amount of liver slices on inhibition of thymidine-^3H incorporation in DNA by given quantity of chalone*

Dose (mg protein)	1,000 A_{600}	DNA specific radioactivity (dpm/1,000 A_{600})*		IF
		Control	Assay	
3.95	180	2059	807	0.61
	286	1590	547	0.66
15.8	182	1914	305	0.84
	255	2049	332	0.84
31.6	108	1862	187	0.90
	195	2238	120	0.95

*DNA by diphenylamine method; values are proportional to number of hepatocytes.

If we suppose that the hepatocytes in the liver slices of the assay flask have n receptor sites, that m of them are occupied by chalone molecules, and that the inhibition of DNA synthesis by the chalone molecules is an all-or-none process:

$$IF = \frac{m}{n}$$

$$1-IF=\frac{n-m}{n}$$

$$K\ (chal) =\frac{(R-chal)}{(R)} = \frac{m}{n-m}$$

$$1+K\ (chal) =\frac{n}{n-m}$$

$$\frac{1}{1-IF} =1+K\ (chal)\,. \qquad [2]$$

Equation 2 is equivalent to equation 1 if the concentration of free chalone in the assay medium is proportional to the amount of chalone added. For that, two conditions must be met: 1) The amount of chalone brought in by the slices must be negligible compared with the exogenous chalone added and 2) the amount of free chalone must be large compared to the amount of bound chalone. We have verified that there is very little chalone in the regenerating liver. The second condition, which supposes that the chalone has a low affinity for its receptor site, can be easily checked. If it is true, the inhibited fraction of DNA synthesis must be largely independent of the amount of slices introduced in the incubation medium; table 4 shows that this is indeed the case.

The same dose-effect relationship was obtained with the most purified liver chalone preparation; it suggests the existence of receptor sites for chalone molecules in the hepatocytes. The chemical nature of this receptor and its location in the liver cell are lines of research that will be pursued as soon as we have a pure liver chalone.

We define the chalone unit as: the amount of chalone necessary to inhibit 50% of the thymidine-^3H incorporation in DNA in our in vitro assay system. The number of chalone units (U) in a fraction of a preparation added to the assay flask can be calculated from the inhibited fraction of DNA synthesis:

$$U=\frac{IF}{1-IF}=\frac{DNA\ sp.rad.(control)-DNA\ sp.rad.(assay)}{DNA\ sp.rad.(assay)}.$$

The relative error in U is minimal when IF = 0.5 and tolerable (< 20%) when IF is between 0.3 and 0.7.

TABLE 5.—*Purification of rabbit liver chalone*

Purification step	Amount of protein (mg)	Total activity (U)	Yield (%)	Specific activity (U/mg)	Purification level
40 ml of 105,000×*g* supernatant	632	253	100	0.4	1
Ethanol precipitation of 40 ml supernatant					
Fraction I	569	57		0.1	
Fraction II	12.5	136	54	10.9	27
Fraction II on Sephadex G–50:					
Fraction A	4.9	16		3.2	
Fraction B	6.6	66	26	10.0	25
UM2 filtration of fraction B:					
Concentrate	5.6	26		4.6	
Ultrafiltrate	0.30	54	21	180	450
Fraction II in UM2 filtration:					
Concentrate	10.6	24		2.3	
Ultrafiltrate	0.59	71	28	120	300

PURIFICATION OF RABBIT LIVER CHALONE

The 105,000 × *g* supernatant of rabbit liver was submitted to fractional precipitation with ethanol. Most of the activity precipitated between 70 and 87% ethanol; the activity not precipitated by 87% ethanol was less than 15% (table 5).

The precipitate was redissolved in water and, in order to concentrate the proteins, the solution was submitted to an ultrafiltration through a Diaflo membrane UM2. Such a membrane, which has pores of 12 nm diameter, retains unit density spherical molecules of a weight above 1,000 Daltons. To our astonishment, the factor inhibitory to liver DNA synthesis was in the filtrate. Thus it is a very small molecule.

With these two steps, we get a 300- to 450-fold purification of the liver chalone with respect to the 105,000 × *g* supernatant. The specific activity, on which we base the estimation of the degree of purification, is the number of chalone units per mg of protein. In the 450-fold purified preparation, 1 unit of chalone corresponds to approximately 5 μg of protein. It is worthwhile mentioning that the supernatant from 1 g of rabbit liver contains approximately 20 chalone units.

The rabbit liver chalone was further purified by chromatography on Sephadex G–15. Most of the experiments reported hereafter were performed with preparations purified only a few hundred-fold (fractional ethanol precipitation followed by ultrafiltration through Diaflo UM2; such purified preparations are referred to as "ultrafiltrates" in contrast to the "supernatants" which are the crude preparations).

NATURE OF RABBIT LIVER CHALONE

The ultrafiltrate was treated with various enzymes and then submitted to ultrafiltration through Diaflo UM2 membranes to get rid of the enzymes before assay on the liver slices. Proteolytic enzymes (trypsin and pronase) destroy the chalone activity; neuraminidase is without action.

The purified liver chalone (ultrafiltrate) is stable at 100° C, but chalone activity disappears from the liver supernatant at 70° C; in the latter case, the chalone probably is coprecipitated with the proteins.

These results suggest that the active principle in the ultrafiltrate prepared from rabbit liver is a small polypeptide.

SPECIFICITY OF LIVER CHALONE

The rabbit liver chalone acts on rat liver; thus there is no species specificity (table 6). In fact, when tested with rat liver slices, the rabbit liver has a higher chalone content than does the rat liver, and this was the reason for choosing the rabbit liver in the experiments that have already been reported. We also have data showing that bovine liver chalone is active on rat liver.

TABLE 6.—*Specificity of liver chalone: Action of different preparations on thymidine-^{3}H incorporation in DNA in slices from various organs of rat*

Preparation	Slices used for assay	DNA specific radioactivity (dpm/1,000 A_{600})		IF
		Control	Assay	
105,000×*g* supernatant, rabbit liver (5 mg protein)	Regenerating rat liver	1,511	378	0.75
105,000×*g* supernatant, rat liver (5 mg protein)	Regenerating rat liver	2,081	1,396	0.33
105,000×*g* supernatant, rat kidney (5 mg protein)	Regenerating rat liver	2,155	2,060	0.04
105,000×*g* supernatant, cow endometrium (10 mg protein)	Regenerating rat liver	1,844	1,686	0.09
UM2 ultrafiltrate from rabbit liver (1 chalone unit)	Regenerating rat liver	1,439	728	0.49
	rat spleen	351	324	0.08
	rat kidney	298	294	0.01

Rat kidney supernatant or cow endometrium supernatant have little effect on DNA synthesis in regenerating liver slices.

The ultrafiltrate from rabbit liver has little action on DNA synthesis in rat spleen or rat kidney; it is also practically without effect on DNA synthesis in rat intestine or HeLa cells. On the other hand, the ultrafiltrate from rabbit liver inhibits DNA synthesis in cultivated normal hepatocytes, in Novikoff hepatoma ascites cells, and in slices from liver hepatoma resulting from treatment with DAB.

The factor, in the ultrafiltrate from rabbit liver, active on regenerating liver DNA synthesis thus qualifies for the name "chalone" according to the definition of Bullough.

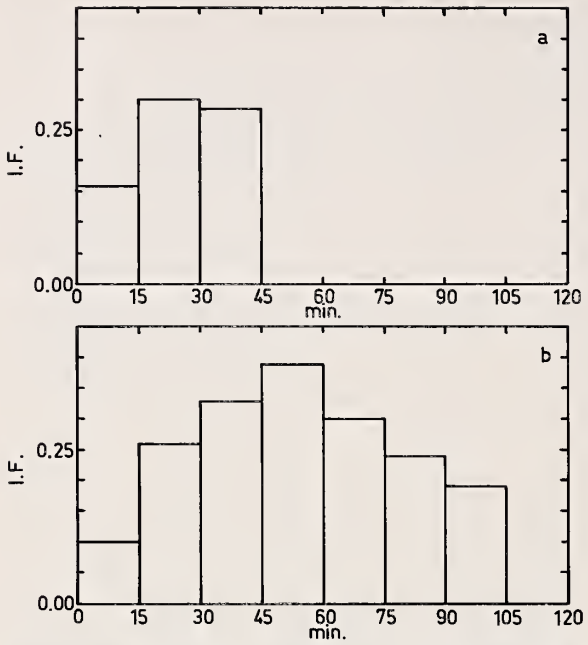
MECHANISM OF ACTION OF LIVER CHALONE

We have investigated the evolution of the DNA inhibition in rat liver slices during the 2-hour incubation. The chalone was added at time 0 of incubation, the thymidine-^{3}H was introduced at various times afterward, and the DNA specific radioactivity in the slices was determined 15 minutes after that. Text-figure 3 shows that, with 0.1 unit of chalone, the inhibition reached a maximum between 15 and 30 minutes after the addition of chalone and that it had completely disappeared after 45 minutes; with 0.3 chalone unit, the maximum occurred somewhat later and the effect lasted longer but eventually disappeared.

We have two things to explain: 1) Why is there a delay in the action of chalone? 2) Why does the action of chalone finally disappear?

Let us answer the second question first. In the control, DNA synthesis proceeds at the same rate during at least 2 hours of incubation. The disappearance of the chalone action after 45 minutes may be due either to the destruction of the chalone or to a loss of sensitivity of the slices. To choose between these alternatives, a chalone preparation was incubated with liver slices for 2 hours (at this time the preparation had no more action on DNA synthesis) and then the incubation medium was transferred onto fresh liver slices. The DNA synthesis was inhibited in the fresh slices as when the first slices were put in contact with the preparation. We conclude that the chalone was not destroyed; after 2 hours of incubation, the slices had become insensitive to the chalone.

We wondered whether the sensitivity of the slices could not be restored. Bullough and Laurence (*8*) have shown that cortisol and adrenaline are necessary for the action of the epidermal chalone. On the other hand, it is known that cortisol and adrenaline inhibit liver regeneration. After 2 hours of incubation of the slices with the chalone preparation (at which



TEXT-FIGURE 3.—Effect of addition of 0.1 unit (*upper*) or 0.3 unit (*lower*) of purified liver chalone ("ultrafiltrate") at time 0 of incubation at 37° C; 20 μCi of thymidine-^{3}H was added at various times later and DNA specific radioactivity was determined 15 minutes afterward. Controls, without chalone, were similarly treated, and the inhibited fraction of thymidine-^{3}H incorporation in DNA (IF) was calculated for each 15-minute interval.

time the chalone had lost its action), we added to the incubation medium cortisol (50 μg) and adrenaline (15 μg) in amounts which were without effect on DNA synthesis in the absence of chalone. The addition of the two stress hormones restored the activity of the chalone. In another experiment, when the two stress hormones were added with the chalone at time 0, the chalone action was prolonged compared to the control without adrenaline and cortisol. These results suggest that the inhibition of liver regeneration observed in vivo after administration of adrenaline or cortisol is mediated by the liver chalone. On the other hand, they indicate that the inhibition of DNA synthesis in the hepatocytes probably has a complex mechanism which needs the synergistic action of several humoral factors.

We also have to explain why there is some delay in reaching a maximal inhibition of DNA synthesis after the addition of the chalone. This delay might be simply due to the time necessary for the chalone to diffuse through the slices and reach the receptor sites in the hepatocytes. We are repeating the experiment using cultivated normal hepatocytes and also isolated cells from the ascitic Novikoff hepatoma; this will tell us what part of the delay must be attributed to the diffusion of the chalone through several cellular layers.

The delay also might be explained if the action of chalone is only a secondary effect; one should then like to identify the first target of the hormone. As a first move to solve this question, we have studied the action of the hepatic chalone on the synthesis of RNA and proteins in slices from regenerating liver. The RNA synthesis is inhibited, but the maximal inhibition occurs later than the maximal inhibition of DNA synthesis; by contrast, the protein synthesis inhibition is immediately at a maximum. I do not think that one can conclude anything about the molecular mechanism of the chalone action from such experiments; this molecular mechanism will be accessible to investigation only when we have the pure liver chalone. We can give a research hypothesis but, first, it is perhaps useful to summarize what is known of the control of DNA replication in bacteria and eukaryotic cells.

The work of Maaløe (*9*) has shown that the replication of *Escherichia coli* chromosomes depends on the synthesis of proteins. When the protein synthesis is inhibited by chloramphenicol, the chromosomes in the process of replicating terminate their replication and there is no initiation of new replication cycles. This shows that the bacterial cell controls very strictly the initiation of replication of their DNA. New data, for instance from the group of Jacob (*10*), suggest what the control might be: A protein, probably a membrane protein, is required for the initiation of DNA replication. Thermosensitive mutants have been found, which, at the nonpermissive temperature, continue the chromosome replication cycles already undertaken but are unable to initiate new replication cycles. Their situation at the nonpermissive

temperature is identical to that of the wild strain in the presence of chloramphenicol. The conclusion seems to be that an initiation protein of high turnover links the initiation of the replication of the bacterial chromosome to the protein synthesis.

The situation is far more complex in eukaryotic cells. However, when stationary cells are stimulated to resume divisions, the same sequence of events is always observed—this has been described for cells of the uterus stimulated by estrogens, cells of the salivary glands stimulated by isoproterenol, cells in culture stationary because of an aged nutritive medium when this medium is replaced by a fresh one, hepatocytes of the remaining lobe after a subtotal hepatectomy, etc. In all cases, a few hours after the stimulus, there is an alternate synthesis of new RNA species and new species of acidic nuclear proteins, leading finally to a signal allowing the cell to cross the G_1–S border (*11*). One can speculate that this signal is the synthesis of an initiation protein needed for the replication of DNA.

The alternate synthesis of new RNA and proteins corresponds to a sequential activation of genes, which resembles what happens during the bacteriophage development in the host bacteria; it is also analogous to the sequential puffing of polytene chromosomes during insect metamorphosis. This sequential activation of genes orchestrates the preparation of the cell for division. Nearly all cell activities are heightened [*see* Bucher and Malt (*1*)]; this leads to an increase of the cellular mass and particularly to a considerable enlargement of the DNA precursor pool. All being ready, the signal to pass into S is given; mitosis follows a few hours after the end of S.

Inhibition of protein synthesis by cycloheximide prevents the G_1 to S transition; it also stops, within 5 minutes, the DNA synthesis in cells already in S (*12*). While in *E. coli* the chromosome is a unique replicon, in eukaryotic cells each chromosome is constituted of several replicons which replicate in orderly fashion during the S phase of the cellular cycle. By analogy with what is known for the bacteria, the action of cycloheximide can be explained if the initiation of the replication of each replicon requires a molecule of initiation protein with a very short half-life.

To come back to the liver chalone, the question is now whether it prevents only the G_1 to S transition or whether, as does cycloheximide, it is also capable of stopping DNA synthesis in cells already in the S phase. In our experiments, the regenerating livers are taken 24 hours after subtotal hepatectomy—i.e., at a time when DNA synthesis is at a maximum and, in our slices, at least 30% of the hepatocytes are in the S phase of the cellular cycle. The chalone action cannot be only on the G_1 to S transition because, with enough chalone, we can observe an immediate and nearly complete inhibition of DNA synthesis. One is thus obliged to conclude that chalone, exactly as cycloheximide, is capable not only of preventing the G_1 to S transition but also can stop the synthesis of DNA in cells already in S. The chalone has no toxic action on cultivated normal hepatocytes; its action is completely reversible.

As for the molecular mechanism of action, I should like to mention two hypotheses for further work.

1) The initiation protein is the receptor site. The inhibition by chalone results from a conformational change similar to that occurring in thermosensitive mutants when the bacteria are brought to the nonpermissive temperature. In the hepatocytes, those replicons which have undertaken replication terminate their replication, but the new initiations are slowed down proportionally to the fraction of initiation protein involved in complex formation with chalone.

2) The chalone might have an action earlier in the sequence of events leading to DNA replication. The action that we observe cannot be on the first step, which occurs a few hours after the partial hepatectomy, because the regenerating liver is taken 24 hours after the operation, but it could be on the synthesis of the initiation protein. In this case, chalone would act as cycloheximide does, the difference being that the action of chalone is tissue specific.

An action of our chalone on the very first steps of the activation leading to cell division

ought also to be explored because Goutier et al. (5) found that a blood factor is able to stop the early nuclear RNA synthesis. The question of whether our factor or a different one is involved in this regulatory mechanism is of the utmost importance.

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Liver Chalone: A Review [1]

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IF EVER there was, liver is an organ in which cell replication is held in check. Whether a chalone will prove to be the sole controlling agent in maintenance of liver tissue mass under normal conditions, during the rapid regeneration after partial ablation, and in control of local cell density are mutually independent questions still to be answered. Another question, also not yet answered, is: What are the exact criteria of chalone action?

The demonstration by Verly et al. (*1*) of a factor extractable from liver with properties close to those that would be expected for a chalone—e.g., from extrapolation of observations on the chalone systems of epidermis or granulocytes—affords further evidence for the soundness of the concepts of Weiss and Kavanau (*2*). Verly's group localized the liver chalone activity to the 70–85% alcohol precipitated fraction of the high-speed supernatant fraction from a water homogenate of whole liver, a process closely analogous to the purification of epidermal chalone in a number of laboratories (*3–5*). The activity was further concentrated on Sephadex G-50 and was eluted at a rate corresponding to a low-molecular-weight compound; subsequent passage of the active principle through a Diaflow UM2 membrane indicated a molecular weight of 2,000 or less. While Boldingh and Laurence considered the molecular weight of epidermal chalone to be 30,000–40,000, Iversen and Elgjo (*6*) provided evidence, from its behavior on Sephadex, that it might be of smaller size. The molecular weight of the material found by Verly's group is close to the 2,000–5,000 firmly indicated for erythrocyte and granulocyte chalones (*7–9*).

The qualitative matching of the biological effects of this presumptive liver chalone with those of chalones already more firmly established is less satisfactory, but not surprisingly so in view of the current lack of agreement as to the mechanism of chalone action. Assays for granulocyte or erythrocyte chalone are based on inhibition of thymidine uptake during a relatively short exposure period (e.g., 5 hr) (*9*); allowing for the diversity of cell types in the test systems, the results suggest that virtually complete inhibition can be obtained, and the assay system used by Verly et al. for liver chalone (inhibition of thymidine uptake into liver slices) is comparable in this respect, since they also can obtain nearly total inhibition in a 2-hour test. In these assays, the chalones are evidently acting in the S phase of the cell cycle.

Earlier studies which are generally regarded as providing evidence for the action of a liver chalone (*10, 11*) have taken depression of mitotic activity after exposure to the inhibitor for a few hours as a measure of the activity—i.e., an effect in G_2. On the other hand, Llanos (*12*) found no inhibition of mitotic activity in regenerating liver in vivo if liver homogenate was administered 5 or 8 hours previously but a marked inhibition if given 12 hours before measuring the effect— i.e., no action in G_2 or in S but effective in G_1. These divergent observations with liver are mirrored in the observations by Elgjo and Hennings (*13*) on chalone extracts of actinomycin D-treated skin. Epidermal chalone preparations are commonly assayed through their ability to depress mitotic rate in a test lasting 4 hours. However, they also lead to a

[1] Presented at the First Symposium of the International Chalone Conference, Brook Lodge, Augusta, Mich., June 5–7, 1972.

depression of DNA synthesis, but this is not observed until about 10 hours after treatment, suggesting an action in G_1 also (*14*). Extracts made from the actinomycin D-treated skin had the same degree of DNA synthesis-inhibiting ability but had no effect on mitosis at 4 hours.

There is insufficient evidence at present to allow the unravelling of the problems raised by these observations, but also there are no objective criteria for rejecting any of the parameters as a measure of chalone action, since each is plausible. (Intuitively, one could expect chalone to act before S phase, but experimentally there is least evidence for this, while the least likely action, in G_2, appears to be mostly firmly established.)

Chopra and Simnett (*11, 15, 16*) have demonstrated tissue specificity for their liver, kidney, and lung chalone effects, and Verly et al. (*1*) have also shown specificity for their liver chalone (compared to kidney) as to both source of extract and target tissue. Speilhoff (*17*) has made preparations of liver and kidney "chalone" by the now classical method of alcohol fractionation and measured their effect in depressing DNA synthesis in vivo in kidney and liver of young rats, 10 hours after administration; he found that they were *not* specific in their action. Speilhoff's preparations were made under sterile conditions, so that the artefactual inhibition of mitosis by bacterial toxins, demonstrated by Elgjo (*18*) as a potential difficulty in measurement of epidermal chalone, cannot be interfering. Volm and Wayss (*19*), using a simple extract of endometrial tissue, found nonspecific effects in a 4-hour test based on measuring DNA synthesis in cultures of liver or kidney cells. They reported that similar nonspecificity may be obtained with extracts of liver and kidney. The extraction procedure is similar to that used successfully, for example by Chopra and Simnett, and the difficulty may lie in the use of cell cultures.

The adrenaline dependence of liver chalone has been demonstrated by Chopra and Simnett in their system, but it remains to be seen whether the rapid effects on DNA synthesis can be shown to be adrenaline sensitive; the need for adrenaline for full action of chalone has so far been demonstrated only in tests based on mitotic inhibition.

The investigations by Tumanishvili and Salamatina (*20*) of the effects of washed nuclear fraction and low-speed supernatants of adult fowl liver on liver development of chick embryos in ova or on regenerating mouse liver (*21*) are not easily reconciled in all aspects with observations on liver chalones made in other laboratories, but it is difficult to believe that their results do not reflect chalone and "antichalone" activities (*7, 22*). Rapid (about 4 hr) inhibition of mitotic activity in the chick embryo liver and a later (about 15 hr) lowering of DNA concentration follow treatment with nuclei, effects which are both detected also 48 hours after treatment. A saline extract of the nuclei similarly rapidly depresses mitotic activity in the embryo liver. Dicker (*23*), using a similar method of preparation for kidney chalone, found the activity, as would be expected, in the supernatant (mitochondria-free), and we (*24*) have been unsuccessful in recent attempts to reproduce Tumanishvili and Salamatina's observations with nuclear and supernatant fractions in chick embryos. In these authors' hands, the low-speed supernatant, which will contain mitochondria and cell fragments, greatly stimulated the mitotic activity in embryo livers, to 60% above control levels at 12 hours, with a second peak rising to 140% at 30 hours, and with a significantly increased DNA concentration at the later time. In our experiments we have studied also thymidine incorporation into chick embryo liver DNA without detecting any significant effects of nuclear or supernatant fractions of liver. It is easily possible, however, that some variation in procedure could account for the unexpected association of chalone activity with the nuclear fraction (*20, 21*).

Teir et al. (*25*) have shown that autolysis of tissue, including liver, releases tissue-specific, but not species-specific factors which stimulate mitotic activity in the corresponding organs. The tissue-specific stimulation of organ cultures of liver confronted with regenerating liver in the same culture (*26*) suggests that stimulators rather than, or as well as, inhibitors are involved under these conditions. Voaden and Lee-

son (*27*) have observed stimulatory activity in the higher molecular weight fraction during their concentration of lens chalone, and Rytömaa (*22*) found that antichalones also elute faster from Sephadex columns than do the corresponding chalones.

Increased liver cell replication can be evoked by plasmapheresis (*28*) but not in ectopic autografts of liver (*29*) or in kidney. To obtain an appreciable response in the liver of the normal partner in cross-circulation with a partially hepatectomized rat, it is necessary for there to be a massive deficit of liver. If the exchange of blood is made exceptionally efficient, the deficient partner still responds as vigorously as in the absence of the cross-circulation with a normal animal (*30*). Neither of these observations can be easily accommodated in a hypothetical mechanism of liver regeneration based solely on a deficiency of chalone. In intact rats, Short et al. (*31*) found that infusion of a "cocktail," designed by them to mimic the increase in free fatty acids in blood as found in partially hepatectomized animals, leads to an increase in the rate of DNA replication in the liver as intense as that following partial hepatectomy, while some other aspects of liver metabolism, such as albumin synthesis, do not change. It remains possible that the response to hepatectomy is not simply the result of chalone deficiency but, if this is so, it is to be hoped that the regenerating liver still can be used to demonstrate chalone-mediated inhibition.

It cannot be said that all observations in the various systems studied which are alleged to evince action of a liver chalone certainly do that, or even that the concept itself is sufficiently defined for such claims to be absolutely meaningful. But, in the way that observations with liver reasonably match those with other tissues, the overall feeling is likely to be that the chalone concept truly reflects reality and that the majority of the observations thought to be on liver chalone are relevant. Nothing will transmute opinions into accepted fact more surely than isolation and characterization of the active materials.

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Regulation of Mitosis in the Embryonic Kidney (*Xenopus laevis*) by Kidney Growth Inhibitor (Chalone) [1]

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SUMMARY—Water-soluble extracts of kidney of adult *Xenopus* or rat contain factors capable of inhibiting cell division in the cultured pronephric kidney of *Xenopus laevis*. The inhibitory action was tissue-specific, since kidney extract had no effect on the explant's epidermis and the extracts of other organs (liver or lung) did not inhibit mitosis in the pronephros. The effect of kidney extract on pronephros appears to be potentiated by very low concentrations of stress hormones (adrenaline and hydrocortisone). An antiserum against the inhibitory kidney extract stimulated mitosis in the pronephros of larval explants. The stimulatory effect is also tissue-specific, since the antiserum actually inhibited mitosis in the larval epidermis. Normal rabbit serum also inhibited mitosis in both tissues, possibly due to a nonspecific toxic effect. It was concluded that the pronephros contains a growth inhibitor which is neutralized by tissue-specific antibody, thus allowing cells to enter mitosis. It is proposed that the regulation of growth by tissue-specific inhibitors, which have been demonstrated in many adult tissues, also occurs in the embryonic kidney of *Xenopus laevis*.—Natl Cancer Inst Monogr 38: 189–196, 1973.

PARTIAL EXTIRPATION of most embryonic or adult organs is followed by compensatory hypertrophy and hyperplasia in the residual tissue. Unilateral nephrectomy in rats results in increased cell proliferation after a latent period of 24 hours and reaches its maximum at 48 hours after the operation (*1*). A similar pattern of regeneration has been found in the embryonic and immature kidney of *Xenopus laevis* (*2*). There is now evidence to suggest that compensatory hyperplasia results from a decrease in concentration of tissue-specific growth inhibitors, caused by partial ablation of the corresponding tissue. The hypothesis is based on the findings that extracts of adult tissue inhibit mitosis of adult organs (in vivo and in vitro) in a tissue-specific manner (*3–5*).

For some years we have been studying the regulation of differentiation and growth in embryonic tissues. There are reasons to believe that growth in both embryonic and adult tissues is regulated by similar mechanisms. Although recent evidence shows that mitosis in adult tissues may be regulated by tissue-specific chalones (*4, 6, 7*), there was no evidence to show whether growth regulation by a similar mechanism occurs in actively proliferating embryonic tissues. We have examined this hypothesis in the pronephric kidney of *Xenopus laevis*. Since preparation of sufficient amounts of extracts from embryonic tissues is not readily accomplished, extracts prepared from adult *Xenopus* or rats were used in our studies. The embryonic kidney responded to the mitotic inhibitory effects of adult kidney extracts. Other experiments have demonstrated indirectly that the embryonic pronephros also contains its own tissue-specific growth inhibitors.

MATERIALS AND METHODS

Our test system was the pronephric kidney of *Xenopus laevis*. This system offered two advantages: 1) In histological sections the pronephros can be readily identified, its tubular structure consisting mainly of epithelial cells; and 2) the

[1] Presented at the First Symposium of the International Chalone Conference, Brook Lodge, Augusta, Mich., June 5–7, 1972.

high rate of cell division in pronephros facilitates the detection of even small inhibitory effects of tissue extracts.

Tissue culture.—Trunk portions of stage 32–33 embryos of *Xenopus laevis* were dissected with the aid of a binocular microscope in Niu-Twitty solution (*8*) supplemented with streptomycin (250 μg/ml in BSS). In addition to the pronephros, explants also contained epidermis, parts of the notochord and neural tube, muscle, and, in some cases, heart. The explants were maintained at room temperature in BSS (approximately 15 minutes) until the cut surface had healed by migration of epidermis. Then they were rinsed twice (10 min each time) in balanced salt solution and finally incubated in groups of five or six in fused silica dishes containing 2 ml of BSS. At stage 32–33, the explants still contain enough yolk platelets to permit development to continue without the addition of extra nutrients. All cultures were maintained at 25–26° C for 2–3 days.

Preparation of tissue extracts.—Tissue extracts were prepared from *Xenopus laevis* or rats. Adult animals were killed instantaneously, and excess blood was removed from the organs by perfusion of the entire animal with a dilute sodium citrate solution (1.9 mg/ml in isotonic saline) administered through the ventricle. The kidney, liver, and lung were removed and weighed. Particular attention was paid to removing all adrenal tissue from the kidney. All subsequent extraction procedures were carried out below 4° C. The organs were thoroughly washed with saline, cut into small pieces, and homogenized in a glass homogenizer with approximately 3 times their volume of BSS. The homogenate was centrifuged in a refrigerated high-speed centrifuge (25,000 rpm) for 20 minutes; the clear supernatant was removed and freeze-dried. The freeze-dried sample of tissue extracts could be stored at −70° C for at least 4 months without significant loss of activity. Just before use, the freeze-dried extract was dissolved in distilled water. The concentration of extracts was expressed as milligrams of dry weight of the original tissue used to prepare the amount of extract added to 1 ml of the culture medium.

Preparation of kidney antiserum.—Antiserum against kidney extract was prepared by immunizing adult rabbits according to a method described elsewhere (*9*). Each rabbit received four series of injections at intervals of 2 weeks. For the first series, a quantity of extract equivalent to 0.3 mg of protein (determined by the biuret method) was made up to 3 ml with isotonic saline, emulsified with an equal volume of Freund's complete adjuvant, and injected intramuscularly at four different sites. For the second and third series of injections, the dose was increased to 3 mg of protein, emulsified with Freund's adjuvant. The fourth series of booster injections consisted of 3 mg of protein suspended in isotonic saline. One week after the final injection, 20 ml of blood was drawn from the marginal vein of each rabbit. The blood withdrawn from each rabbit was processed separately. The blood was stirred to remove fibrinogen, incubated at 37° C for 3 hours, and centrifuged at 4,500 rpm for 30 minutes. The clear supernatant (kidney antiserum) was stored at −70° C. Normal rabbit serum was prepared by the same method using samples of blood taken from nonimmunized rabbits.

Estimation of mitotic index (MI).—Before the termination of each experiment, test substances (tissue extract, hormone, antiserum, or normal rabbit serum) and Colcemid (0.025 mg/ml) were added to the culture. Subsequently, the cultures were fixed in Worcester fluid, dehydrated in Cellosolve, embedded in paraffin wax, sectioned serially at 5μ and stained in Weigert's hematoxylin for histological examination. In each explant, the number of arrested metaphases was counted in a sample of 1,000 to 1,500 nuclei for pronephros (in some cases also for epidermis), and the mean MI and standard deviation were calculated for each group. The value of MI for each group was expressed as the number of arrested metaphases per 10^5 cells. The significance of the effect of a treatment was evaluated by Student's t test.

RESULTS

Effects of Different Tissue Extracts on MI in Pronephros

After 3 days of incubation of kidney cultures, tissue extracts (18 mg/ml, kidney, liver, or lung)

TABLE 1.—*Effect of different adult organ extracts on mitotic index in pronephros of larval explants*

	Untreated	Treated*		
		Kidney extract	Liver extract	Lung extract
MI†, mean ± SD	2,363±928	1,113±685	2,289±978	2,958±1,401
% depression	—	53	3	—
P	—	<0.05	<0.09	—

*Concentration of extracts, 18 mg/ml (dry weight of original tissue from which the extract was prepared).
†Number of arrested metaphases per 10^5 cells during 4-hour period of Colcemid treatment.

TABLE 2.—*Effects of stress hormones and adult kidney extract on mitotic index in pronephros and epidermis of larval explants*

Condition*	Pronephros		Epidermis	
	MI, mean ± SD	% depression	MI, mean ± SD	% depression
Untreated	2,258 ± 415		4,534 ± 969	
Kidney extract	1,088 ± 367	52†	3,898 ± 742	14‡
Stress hormones	965 ± 318	57†	1,886 ± 435	58†
Kidney extract plus stress hormones	221 || ± 189	90† 77§	2,153 ± 498	53†||

*Final concentrations of kidney extract, 18 mg/ml; stress hormones, 0.0025 μg/ml.
†Depression significant (P <0.001) compared with untreated control.
‡Depression not significant compared with untreated control.
§Depression significant (P <0.001) compared with stress hormones alone.
||Depression not significant compared with stress hormones alone.

and Colcemid were added. The control cultures received only Colcemid. Kidney extracts inhibited mitosis by 53% ($P<$ 0.05) while liver or lung extracts had no signficant effect (table 1).

Effects of Stress Hormones (Adrenaline and Hydrocortisone) and Kidney Extract on MI in Pronephros

For some tissues it has been demonstrated that the tissue-specific effect of growth inhibitors is potentiated by stress hormones (*10*). Although, in the present work, kidney extract by itself depressed cell division by 53%, the possible synergistic effect of stress hormones with kidney extract was examined in the following experiment.

Four groups of cultures were set up as follows: 1) untreated, 2) kidney extract only (18 mg/ml), 3) adrenaline and hydrocortisone (0.0025 μg/ml each), and 4) kidney extract (18 mg/ml) and adrenaline and hydrocortisone (0.0025 μg/ml each). All cultures received Colcemid at the same time and were fixed 4 hours later. The addition of kidney extract alone inhibited mitosis by 52%, and stress hormones alone gave a depression of 57% (table 2). In combination, kidney extract and stress hormones depressed cell division by 90%. Analysis of variance showed that the effect of either kidney extract or stress hormones was highly significant ($P<0.001$). These results suggested that stress hormones may potentiate the inhibitory effect of kidney extract, although in this kind of experiment such effects may not show clearly. One can argue that the inhibitory

TABLE 3.—*Effects of different concentrations of stress hormones separately and in combination on MI in pronephros of larval explants*

Concentration (μg/ml)	Adrenaline		Hydrocortisone		Adrenaline + hydrocortisone	
	MI, mean ± SD	% depression	MI, mean ± SD	% depression	MI, mean ± SD	% depression
Control	1,819±340					
0.00025	1,494±705	18	1,956±916	—7	1,710±289	6
0.0025	1,450±654	20	1,283±283	29	918±159	50
0.025	1,135±531	38	1,352±942	26	704±389	61

effects of both factors are additive, since both the hormones and the kidney extract inhibited mitosis significantly when added separately.

However, this point was clarified by an indirect method in which very high concentrations of hormones or kidney extracts were added separately. If the hormones acted synergistically with the kidney growth inhibitor, their combined effect should be greater than the maximal inhibition produced by them separately. Results of these experiments are presented in tables 3 and 4. It was found that a tenfold increase of stress hormones or an approximately fivefold increase of kidney extract did not produce more inhibition than produced by them together at lower concentrations (tables 3 and 4).

In a further series of experiments the effects of kidney extract, adrenaline, and hydrocortisone were tested with the factors being added singly, in pairs, and all together after a 2–day culture period. When added separately, adrenaline or hydrocortisone had no inhibitory effect, while together they depressed mitosis in the pronephros by 43% (table 5). When combined with kidney extract, adrenaline or hydrocortisone had no potentiating effect. However, when the three factors were added together, the inhibition of cell division in the pronephros reached a maximum of 84%. Thus, it appears that, if stress hormones form a complex to potentiate the effect of kidney extract, both adrenaline and hydrocortisone must be present in the culture fluid. Similar results were obtained for epidermis of the explants except that, in this case, kidney extract with or without hormones had no effect on cell division.

Effects of Antikidney Serum or Normal Rabbit Serum on MI in Pronephros

The experiments described above demonstrated that cell division in the embryonic kidney of *Xenopus laevis* may be regulated by growth inhibitors which are tissue-specific but not species-specific. However, we do not yet know the chemical nature of the inhibitory factors, nor has it been established whether these factors are present in the pronephros itself. Both these points were examined by using an indirect

TABLE 4.—*Effects of different concentrations of kidney extract on MI in pronephros of larval explants*

	Concentration (mg/ml)					
	Control	1	3	9	27	81
MI, mean ± SD	2,134 ± 362	1,327 ± 1007	1,272 ± 387	1,032 ± 316	784 ± 214	747 ± 50
% depression		38	40	52	63	65

TABLE 5.—*Effects of kidney extract and stress hormones separately and in combination on MI in pronephros and epidermis of larval explants*

Condition*	Pronephros		Epidermis	
	MI, mean ± SD	% depression	MI, mean ± SD	% depression
Control	2,228±590	—	3,283±469	—
Adrenaline	2,241±1265	—	2,578±550	21
Hydrocortisone	1,955±895	12	2,712±736	17
Adrenaline + hydrocortisone	1,275±782	43	1,722±641	48
Kidney extract	1,220±275	45	2,254±990	31
Kidney extract + adrenaline	1,421±627	36	3,135±696	5
Kidney extract + hydrocortisone	1,449±483	35	2,389±462	27
Kidney extract + adrenaline + hydrocortisone	364±239	84	1,211±362	57

*Final concentrations: adrenaline, 0.0025 μg/ml; hydrocortisone, 0.0025 μg/ml; and kidney extract, 18-20 mg/ml.

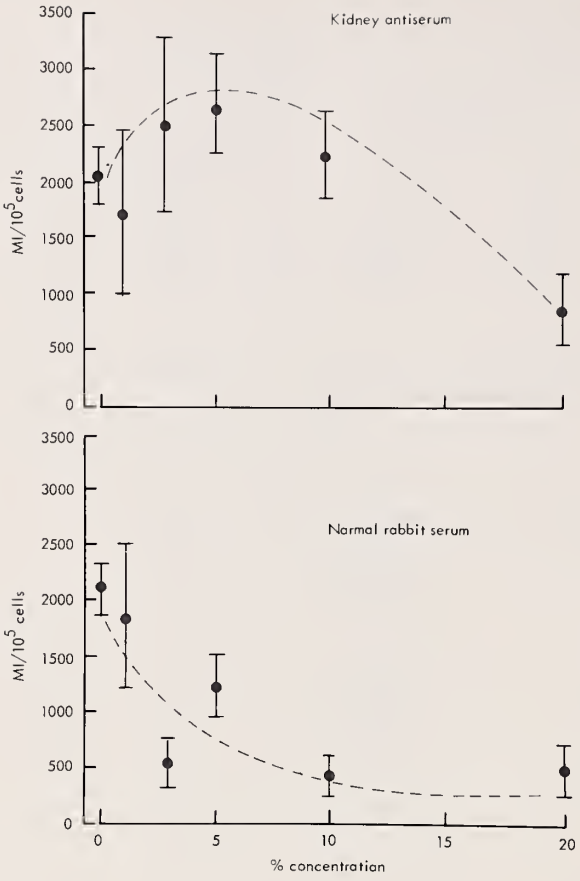
method, since it is difficult to prepare extracts from small organs such as the pronephros. If, as suggested by studies on other organs (*11*), the inhibitor is an antigen, it should be possible to prepare an antibody against it. The effect of this antibody on the pronephros would be to neutralize the endogenous inhibitor and thereby stimulate mitosis.

Antikidney serum or normal rabbit serum was added at various concentrations to the pronephros cultures and MI was calculated in the pronephros after a 4-hour treatment. While 1% antiserum did not have any significant effect on cell proliferation in the pronephros, a concentration of 3% stimulated mitosis by 21% (text-fig. 1). The maximal stimulatory effect was achieved with 5% antiserum, which stimulated cell division by 29% ($P<0.02$) when compared with the control pronephros. There was no significant difference between the values of MI in the control pronephros and in those treated with 10% antiserum; 20% antiserum appeared to have some toxic effect on the pronephros, since it gave a 59% depression ($P<0.02$).

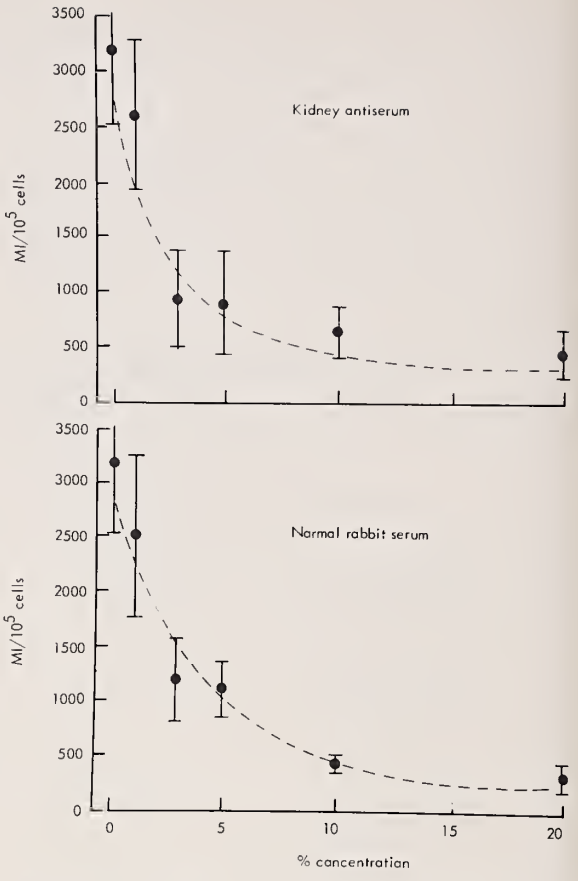
In contrast to its effect on pronephros, all concentrations of antiserum inhibited cell division in the epidermis of explants (text-fig. 2), which suggests that antiserum probably has some nonspecific toxic effect.

Unlike antiserum, normal rabbit serum inhibited mitosis unselectively in the pronephros and in the epidermis.

These data suggested that, with the exception of the effect of antiserum on pronephros (where



TEXT-FIGURE 1.—Effects of different concentrations of kidney antiserum and normal rabbit serum on mitotic index (MI) in pronephros in vitro.



TEXT-FIGURE 2.—Effects of different concentrations of kidney antiserum and normal rabbit serum on mitotic index (MI) in epidermis in vitro

it stimulated mitosis), both normal rabbit serum and antiserum had a toxic effect on tissues of embryonic explants. It is not clear, however, whether the stimulatory effect of the antiserum was due to the immunization of the donor rabbit or whether it was merely a reflection of naturally occurring differences between individual donors. For this reason, another antiserum was prepared using a different kidney extract and different rabbits. Normal rabbit serum was also prepared from two other rabbits. The effects of this antiserum and normal serum on the MI in the pronephros and epidermis were examined. The results of this experiment were similar to those obtained in the first experiment: 3 and 5% antiserum had a stimulatory effect on the MI in the pronephros while all concentrations of antiserum or normal rabbit serum inhibited cell division in the epidermis. It therefore appears that the effects of the antiserum preparations are related to the course of immunization given to the donor rabbits.

DISCUSSION

The results presented here show that extracts of kidney from rat or *Xenopus* inhibit mitosis specifically in the pronephric kidney of *Xenopus laevis*. The active factor or factors are water soluble and are present in the clear supernatant of a centrifuged homogenate of cells. It is tissue-specific in both origin and effect.

Bullough and Laurence (*10*) have reported

that the inhibitory effect of the epidermal chalone is potentiated by adrenaline and hydrocortisone. In the present studies, the stress hormones at low concentrations appeared to augment the inhibitory effect of the kidney extract. The mechanism by which chalone and stress hormones interact is unknown. Two possibilities have been suggested for the epidermis (*10*): 1) The active antimitotic agent may be an unstable chalone-adrenaline complex and hydrocortisone acts to stablize this complex; and 2) the hormones (at least the hydrocortisone) act by their action on the cell membrane and are able to delay the breakdown or the release of the complex from the cell.

Little is known regarding the influence of chalone on the cell cycle. Most studies (*4, 12*) indicate that the active factors influence cell division by an effect on the G_2 phase, since the inhibitory action was demonstrated within 4 hours (a period shorter than the duration of G_2) after the addition of tissue extracts. Other investigators have shown that at least epidermal chalone may also influence other phases of the cell cycle. Iversen (*6*) showed that, besides inhibiting mitosis, the epidermal chalone depressed the incorporation of thymidine-^{3}H in epidermis. More recently, Elgjo (*13*) demonstrated that the inhibitory effect of chalone may be greater in G_1 than in G_2. The fact that mitotic inhibition by kidney extract or stimulation by kidney antiserum was obtained in the 4–hour period after administration of the test substances suggests that at least in the present experiments the inhibitory or stimulatory factors acted by modifying the movements of cells from G_2 to M. We do not yet know if the kidney growth inhibitor influences other phases of the cell cycle.

Partial extirpation of the pronephros or immature mesonephric kidney of *Xenopus laevis* is followed by an increase in the mitotic rate in the remaining tissue (*2*). The present results support the earlier conclusion that compensatory growth in the kidney is probably due to a temporary decrease in the systemic concentration of a tissue-specific growth inhibitor. Control of cell production through a negative-feedback mechanism mediated by tissue-specific inhibitors may thus play an important part not only in adult tissue homeostasis but also in the regulation of growth in the developing embryo.

The gradual decrease of cell division in embryonic tissues as they approach the fully differentiated state may be understandable on the assumption that embryonic cells at first do not produce any chalone. As the tissues are determined, probably as a result of certain inductive mechanisms (*14*), each cell type begins to produce its own inhibitor. During the early stages of development, the concentration of the inhibitor may not be sufficient to produce any significant inhibition of cell division. The gradual increase in functional activity, which is believed to be accompanied by a similar increase in the local and systemic concentrations of the inhibitor, may result in a gradual decrease in mitotic activity. In the adult animal the active concentration of the inhibitor is such that it only allows that cell division which is essential for the replacement of cells lost through degenerative and other processes.

Although it has been shown that adult kidney extracts inhibit mitosis in embryonic kidney, the presence of tissue-specific chalones in pronephros has been demonstrated only by an indirect method. It was assumed that, if the kidney growth inhibitor were a glycoprotein [as has been found for epidermis (*11*)], it should be possible to prepare an antiserum against it. The effect of such antiserum, it was argued, would be to neutralize the endogenous antimitotic factor present in the intact kidney tissue and hence to stimulate mitosis. The results obtained in the present series of experiments indeed show that immunization of rabbits with mitosis-inhibiting kidney extract causes their serum (antikidney serum) to have a stimulatory effect on mitosis in pronephros of cultured larval explants. The data therefore support the view that the embryonic kidney contains a tissue-specific mitosis-inhibiting factor which may be an antigen.

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Self-Limitation of Ascites Tumor Growth: A Possible Chalone Regulation [1]

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SUMMARY—Since most tumor-growth studies have been performed on growing tumors, it seemed of interest to investigate the conditions in the malignant ascites tumor at the growth plateau. In this way, it was hoped to throw light on some of the causes of the balanced growth of the advanced ascites tumor.—Natl Cancer Inst Monogr 38: 197–203, 1973.

IT IS well known that the growth of experimental ascites tumors is characterized by a decelerating growth rate with increasing number of cells. Finally, a plateau phase with zero growth is reached, which means that cell birth equals cell loss. Although the growth rate in a variety of tumors decreases with increasing size, a real plateau phenomenon is rarely observed, probably because the death of the host prevents the tumor from reaching a steady state.

The most obvious causes of the decreasing growth rate of an old ascites tumor are deficient oxygen supply, exhaustion of nutrients, and accumulation of toxic metabolites from dying tumor cells (*1, 2*), but recent studies have revealed that, although these unfavorable conditions undoubtedly influence the growth rate, other factors seem more important in determining the overall growth of ascites tumors (*3–5*).

As a working hypothesis it was assumed that the growth deceleration in the aging ascites tumor is due to a specific negative-feedback mechanism based on an increasing concentration of a humoral inhibitor produced by the increasing number of tumor cells.

It is generally agreed that the growth deceleration in advanced ascites tumor is brought about mainly by a progressive lengthening of the cell cycle and, to a varying degree, by a decrease in the growth fraction, while the cell loss appears to be almost constant except for the terminal period (*6–12*).

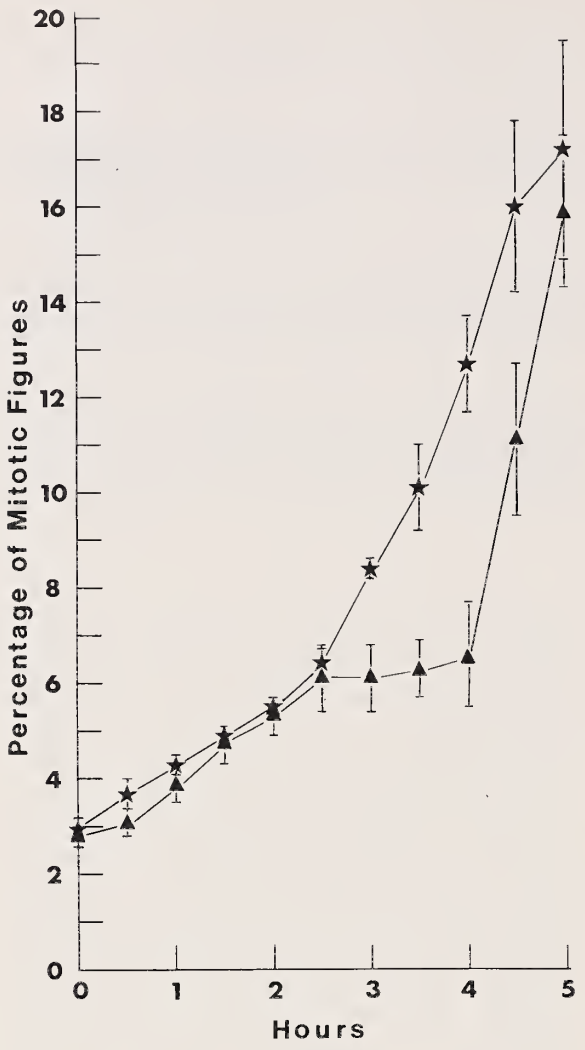
When a large part of the ascites tumor in a mouse is removed by percutaneous puncture in the plateau phase of growth, a marked growth acceleration is demonstrable in the remaining tumor 24 hours after the aspiration (*13, 14*), reminiscent of the recurrent growth seen, for example, after major hepatectomy. Because of the suspended nature of ascites tumor cells, the transmission of information between the individual tumor cells concerning the actual state of development of the ascites tumor is most likely mediated humorally via the intercellular ascitic fluid.

To test this hypothesis, the effect of injection of cell-free ascitic fluid from old ascites tumors on recurrent growth was investigated in a hypotetraploid ascites tumor JB-1: a plasmacytoma carried syngeneically in an inbred strain of mice.

METHODS AND RESULTS

Samples (2 ml) of cell-free ascitic fluid from JB-1 ascites tumors at the growth plateau were injected into mice bearing JB-1 ascites tumors in recurrent growth (24 hr after aspiration), and

[1] Presented at the First Symposium of the International Chalone Conference, Brook Lodge, Augusta, Mich., June 5–7, 1972.



TEXT-FIGURE 1.—Accumulation of JB-1 ascites tumor cells in mitosis after intraperitoneal injection of Colcemid at zero hour. ★—★ = recurrent growth (i.e., 24 hr after aspiration of as much as possible of the ascites tumor from mice inoculated 10 days earlier with the ascites tumor cells). ▲—▲ = recurrent growth with addition at zero hour of 2 ml of cell-free ascitic fluid obtained from mice bearing JB-1 ascites tumors in the plateau phase of growth. Reproduced from the *European Journal of Cancer*—Pergamon Press—with kind permission of publishers.

simultaneously the mice received an intraperitoneal injection of Colcemid. By means of repeated aspirations of very small samples of the ascites tumor, the accumulation of cells in mitosis was followed on smears for 4–5 hours after the injection of Colcemid (*15*).

For the first 2½ hours, the increase in the number of mitoses was identical in the controls subjected only to aspiration and in the mice given injections of ascitic fluid (text-fig. 1). From 2½ to 4 hours after the injection, there was a complete cessation of the flux of cells to mitosis, indicating that all the cycling cells had been blocked about 2½ hours ahead of the mitotic phase.

Four hours after injection of ascitic fluid a sharp rise in the accumulation curve was observed, probably due to the wave of cells which had been accumulated before the block and indicating a rapid turnover of the inhibitor. Apparently no or at least very few cells were lost during the inhibition.

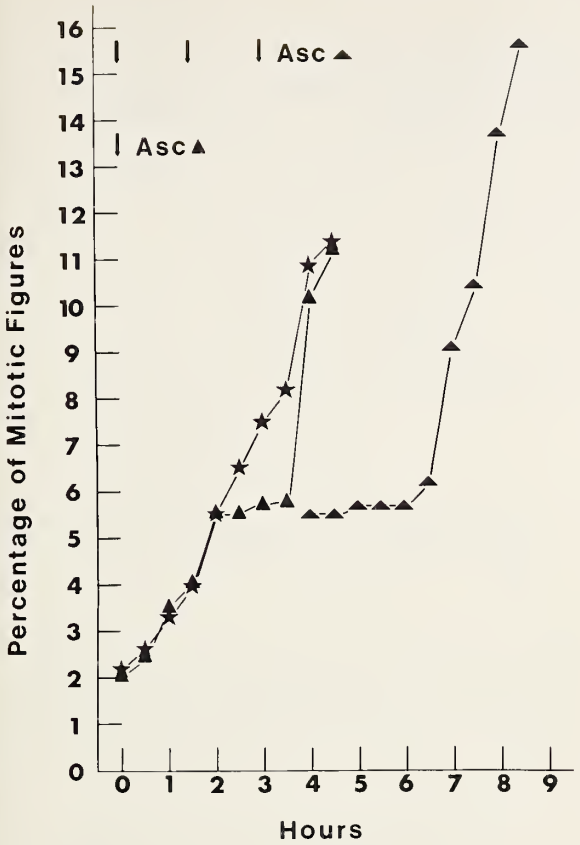
Repeated injections of the acellular ascitic fluid at 1½-hour intervals were followed by prolonged arrest of the tumor cells, corresponding to the number of injections (text-fig. 2).

The duration of $G_2+\frac{1}{2}M$ of the ascites tumor in recurrent growth was estimated to be 3–4 hours, by means of the labeled mitosis method. The interval of about 2½ hours between the injection of the ascitic fluid and cessation of entry of tumor cells into mitosis suggests that the block of the cell cycle occurs in the beginning of the G_2 phase.

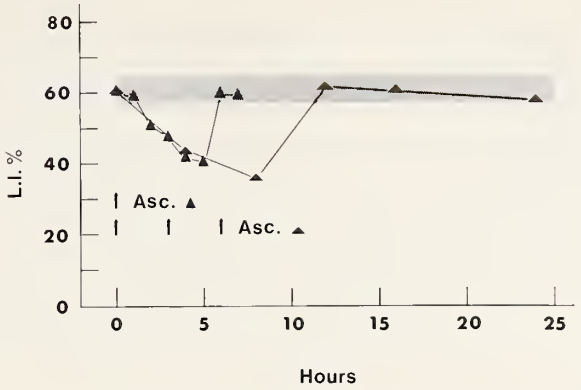
It is, however, the prevailing opinion that control of growth is usually a control of the initiation of DNA synthesis (*16*).

To investigate the possibility that the cell-free fluid of the old ascites tumor induces arrest of cells in the presynthetic phase, the thymidine-^{3}H labeling index was determined in JB-1 ascites tumors in recurrent growth in the hours after intraperitoneal injection of 2 ml of ascitic fluid (*15*). After about 1 hour, the labeling index was found to be constantly decreasing for about 4 hours before returning to normal, and repeated injections extended the effect (text-fig. 3).

Thus the cell-free ascitic fluid seems to induce inhibition of the cells in both the G_1 and the G_2 phase of the cell cycle. On the basis of these experiments, an accumulation of tumor cells may be expected in the G_1 and the G_2 phase in the JB-1 ascites tumor at the plateau. In some nor-



TEXT-FIGURE 2.—Accumulation of JB-1 ascites tumor cells in mitosis after intraperitoneal injection of Colcemid. ★—★ = recurrent growth; addition of Colcemid at zero hour. ▲—▲ = recurrent growth; addition, at zero hour, of Colcemid and 2 ml of cell-free ascitic fluid from mice bearing fully developed ascites tumors. ▲—▲ = recurrent growth; addition of 2 ml of cell-free ascitic fluid at 0, 1½, and 3 hours and addition of Colcemid at 4 hours. Reproduced from the *European Journal of Cancer*—Pergamon Press—with kind permission of publishers.



TEXT-FIGURE 3.—Thymidine-^{3}H labeling index of JB-1 ascites tumor cells. ▒ = range of labeling index for tumors in recurrent growth. ▲—▲ = recurrent growth; addition at zero hour of 2 ml of cell-free ascitic fluid from JB-1 ascites tumors in their plateau phase of growth. ▲—▲ = recurrent growth; addition of 2 ml of cell-free ascitic fluid at 0, 3, and 6 hours. Reproduced from the *European Journal of Cancer*—Pergamon Press—with kind permission of publishers.

mal tissues, noncycling cells have been demonstrated in both G_1 and G_2 (*17–19*).

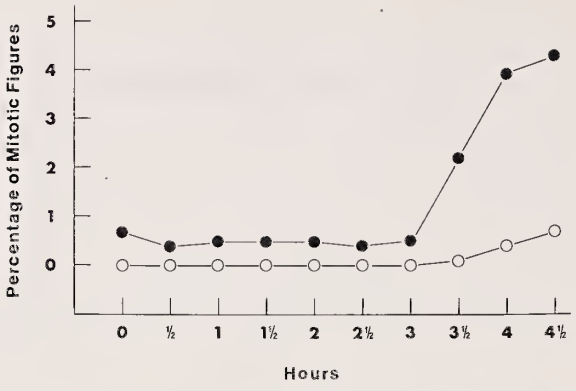
According to the literature, there are different opinions about G_0 cells with G_2 DNA values in ascites tumors. A resting subpopulation of tumor cells with 4N DNA was demonstrated in the sarcoma 180 ascites tumor by DeCosse and Gelfant (*20*). Similarly, Frindel et al. (*8*) found that the percentage of cells in the G_2 phase increased with the age of the NCTC 2472 ascites tumor.

However, Lala and Patt (*21*) and Peel and Fletcher (*22*) found that the G_0 population of old Ehrlich ascites tumors had G_1 DNA values, and no arrest of cells could be demonstrated in the G_2 phase.

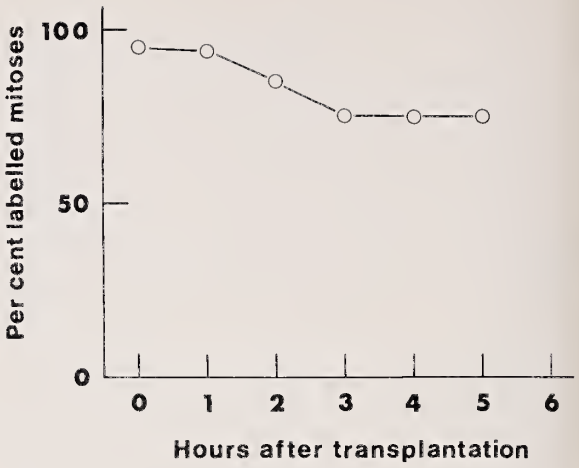
When tumor cells from old ascites tumors are transplanted intraperitoneally into new hosts, the mitotic index usually decreases almost to zero during the first hour after transplantation. Three to four hours later, a marked increase occurs in the number of mitotic cells. According to Lala and Patt (*21*), the increase of the mitotic index is due to a lag in the shortening of mitosis relative to that of other stages during the initial readjustment of the cell cycle, while Wiebel and Baserga (*10*) explained the findings by a transient delay of cells in G_2 followed by a rapid acceleration.

If the plateau tumors are prelabeled with thymidine-^{3}H in the donors immediately before transplantation, it is seen that unlabeled cells are responsible for the increase in the mitotic index 3–4 hours after transplantation (text-fig. 4). The unlabeled cells represent cells which, at the time of transplantation, were in the G_2 phase.

In a comparison of the accumulation curve of the old tumor cells in the donors with that obtained after transplanting the old cells to new



TEXT-FIGURE 4.—Transplantation to new hosts at zero hour of old JB-1 ascites tumor cells which, immediately before transplantation, were prelabeled in the donors for 15 minutes with thymidine-^{3}H. Accumulation of the tumor cells in mitosis was determined in the new hosts by means of Colcemid injected at zero hour. ●—● = unlabeled mitosis. ○—○ = labeled mitosis.



TEXT-FIGURE 5.—Labeled mitoses of JB-1 ascites tumor cells after inoculation, into new hosts, of 100×10^6 prelabeled cells for 20 hours with thymidine-^{3}H every 4th hour.

hosts for periods corresponding to the duration of their respective G_2 phases, it was seen that about twice as many mitotic cells had accumulated in the transplanted animals as in the donors. Although a transient delay of cells in G_2 following transplantation may be part of the explanation, this probably means that in the old tumor a fraction of the cell population was arrested in the G_2 phase and was released when the cells were transplanted to the new hosts.

When ascites tumors at the plateau are repeatedly labeled with thymidine-^{3}H for a period corresponding to one generation time, all cells in cycle are supposed to be labeled and most of the cells outside the cycle are probably unlabeled. Within a period corresponding to G_2 after transplantation of repeatedly labeled old tumor cells to new hosts, a marked decrease in the percentage of labeled mitoses was seen (text-fig. 5), which means that, in the old ascites tumor, some cells were probably resting in G_2 ready for mitosis.

Furthermore, direct cytophotometric investigations revealed that the proportion of the tumor cells which had G_2 DNA values increased with the age of the JB-1 ascites tumor.

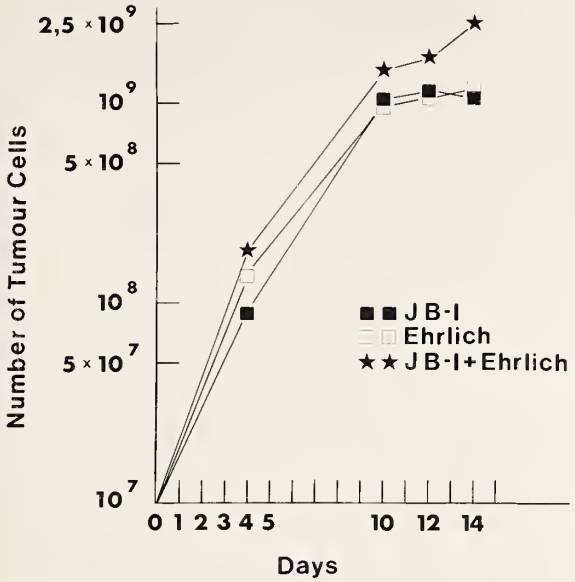
As to the possible accumulation of cells in the G_1 phase of the advanced ascites tumor, it was found that transplantation of old ascites tumor cells to healthy recipients almost immediately gave rise to an increase in the number of cells which entered the S phase, indicating a concentration of cells in the presynthetic phase in the old tumor.

Thus it is suggested that increasing age or size of the ascites tumor is followed by inhibition of the cells in the G_2 as well as in the G_1 phase of the cell cycle.

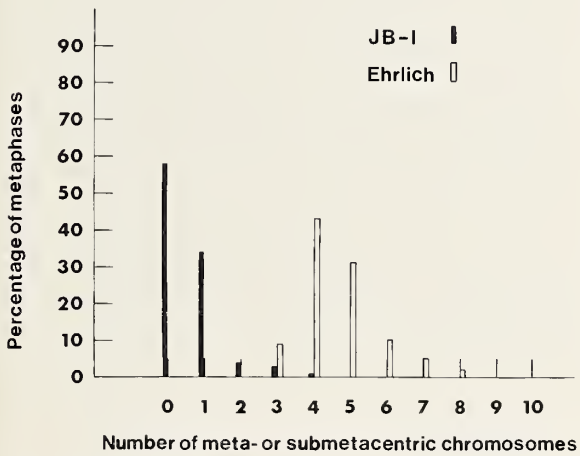
When the total number of cells is determined at different stages of the growth of JB-1 and other hypotetraploid ascites tumors, it appears that, after a rapid initial growth, the tumors reach a maximum level of about 1×10^9 cells. About twice that number of cells was reached when the JB-1 ascites tumor was inoculated intraperitoneally simultaneously with a hypotetraploid line of the Ehrlich ascites tumor in the same mouse (text-fig. 6). A study of the chromosomal pictures of the JB-1, the Ehrlich, and the mixed JB-1 and Ehrlich ascites tumor cells revealed that the two tumors participated almost equally in the combined growth (text-figs. 7, 8).

Similar results were obtained by Brown (*4*), who inoculated the Ehrlich ascites tumor simultaneously with the Crocker ascites sarcoma 180 into the same mouse.

These double-inoculation experiments seem to indicate that oxygen deficiency, nutritional



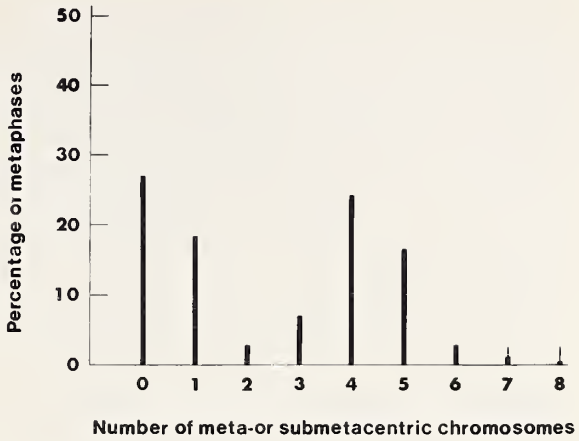
TEXT-FIGURE 6.—Growth curves of ascites tumors (semilogarithmic). Reproduced from the *European Journal of Cancer*—Pergamon Press—with kind permission of publishers.



TEXT-FIGURE 7.—Percentage of metacentric and submetacentric chromosomes in mitotic figures of JB-1 and Ehrlich ascites tumor cells. Reproduced from the *European Journal of Cancer*—Pergamon Press—with kind permission of publishers.

problems, and accumulation of toxic metabolites play only minor roles in the occurrence of the plateau phenomenon of the ascites tumor.

The assumption that the deceleration of growth in the old ascites tumor is due to a specific inhibition is also supported by the observation that no inhibition of tumor cells occurred in G_1 and G_2 when Ehrlich ascitic fluid was injected into JB-1 ascites tumors, or vice versa.



TEXT-FIGURE 8.—Percentage of metacentric and submetacentric chromosomes in mitotic figures of cells in the mixed JB-1 and Ehrlich ascites tumors simultaneously inoculated into the same mouse. Reproduced from the *European Journal of Cancer*—Pergamon Press—with kind permission of publishers.

Similar results were obtained with the JB-1 ascites tumor and a hypotetraploid ascites tumor, HB, originally derived from a spontaneous mammary carcinoma.

DISCUSSION

It has recently been reported that the presence of an advanced ascites tumor does not influence the proliferation of the myeloid-erythroid precursor cells of the bone marrow (*23*). It was concluded that the decreasing growth rate of the aging ascites tumor must be attributed to probably nonspecific changes in the local ascites environment, and that these inhibitory substances possibly do not circulate in the host blood in a concentration high enough to retard cell proliferation in renewing tissues, such as the bone marrow.

In parabiotic mice, however, we have found that recurrent growth of a JB-1 ascites tumor in one of the mice was almost completely prevented when the parabiotic partner had a JB-1

ascites tumor at the plateau (*24*). It is suggested that recurrent growth is prevented by passive transfer of the inhibitor or inhibitors, via the blood, from the old tumor to the parabiotic partner bearing the aspirated ascites tumor. Thus, it is possible that the absence of inhibition of the bone-marrow cells in mice with advanced Ehrlich ascites tumors is a question of specificity rather than concentration.

By means of ultrafiltration of the cell-free ascitic fluid it has been possible to separate two fractions, one with a molecular weight between 1,000 and 10,000 and inhibiting the cells in the G_2 phase, and one with a molecular weight between 10,000 and 50,000 with G_1-inhibiting properties (*25*).

Probably, the effects are due to substances of protein nature, since the inhibiting properties of the two fractions completely disappeared after treatment with a proteolytic enzyme.

DeCosse and Gelfant (*26*) demonstrated that, in the Ehrlich ascites tumor, immune inhibition may restrain ascites tumor cells from cycling and may block them in G_2 and apparently this block was neutralized by immunosuppressive agents.

The low molecular weight of the inhibitors in the ascitic fluid, the production of ascitic fluid with inhibiting properties in mice treated with sublethal irradiation, and the fact that water extracts of old ascites tumor cells contain substances with identical inhibitory properties seem, however, to exclude the possibility that immune reactions should be responsible for the G_1 and the G_2 inhibition of the cell cycle in the JB-1 ascites tumor.

Although it must be taken into consideration that the tumor-bearing organism represents a very complex situation, it seems that the suggested hypothesis of increasing concentration of specific humoral growth inhibitors arresting the cells in the G_1 and the G_2 phase of the cell cycle and dependent on the cell number might be at least a partial explanation of the growth deceleration in the ascites tumor.

When these observations on the ascites tumors are taken together with our present knowledge of the normal chalones, several similarities are apparent—i.e., the restraint of cells in G_1 and G_2 (*27, 28*), the specificity of the reactions (*29*), and the almost identical molecular weights of the inhibitory substances (*30, 31*). Thus the criteria for a normally acting chalone control in the ascites tumor seem to be fulfilled.

Several investigators have concluded that a seriously reduced intracellular concentration of chalone due to increased loss through the cell membrane (*32, 33*) may be a common characteristic of malignant tumors.

However, mathematical analyses of a variety of tumors have suggested that tumor growth may be described as an exponential process limited by an exponential retardation, which might in turn be explained by assuming an increasing concentration of an inhibitor produced by an increasing number of cells.

It is conceivable that, during the malignant transformation of a tissue, a new set of genetically determined information concerning the physiological upper limit of the size (*34, 35*) has been acquired.

It might be speculated whether we are dealing with some kind of hypertrophied tissue which has partially escaped regulation by the tissue of origin owing to an increased loss of normal chalone through the cell membrane, or whether it is a question of a tissue subjected to a complete regulation of its own which, on account of a certain relationship with a normal tissue, is accidentally more or less sensitive to the chalones of this tissue.

It is possible that supplementary investigations of the conditions in other old tumors and the content of chalone during normal tissue regeneration may contribute to the elucidation of these problems.

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Influence of Adenosine Diphosphoribosylation of Histones on the Replication of Human Cells [1, 2]

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SUMMARY—Currently available evidence suggests that ADP-ribosylation of nuclear proteins might be a normal control mechanism of the eukaryotic cell for preventing unscheduled initiation of DNA synthesis and hence controlling mitosis. To investigate the physiologic significance of this theory, the activity of polyadenosine diphosphoribose (poly-ADPR) polymerase was studied during various stages of a synchronized human cell cycle. There is no evidence at this time that poly-ADPR polymerase is involved with a chalone mechanism in HeLa cells—Natl Cancer Inst Monogr 38: 205–211, 1973.

POLYADENOSINE diphosphoribose (poly-ADPR) polymerase, an enzyme localized in nuclei of many human tissue culture cell lines as well as of other eukaryotic organisms, catalyzes transfer of the ADPR moiety of NAD covalently to histones. ADPR units then are elongated on the histone; the linkages between adjacent ADPR units are by a ribose-1′ to ribose-2′ glycosidic bond (*1–3*). Poly-ADPR polymers of 10–80 successive units have been reported to have been isolated from nuclei in vitro. In vitro, the enzyme has a unique requirement for DNA for the chain-elongation step. The poly-ADPR polymer has been shown to be rapidly turning over in nuclei; recently, there has been reported the isolation of an enzyme which cleaves the ribose-ribose linkage of poly-ADPR (*4*). Furthermore, it has been reported that cyclic AMP inhibits the degradation of ADPR attached to histones (*5*).

Our interest in this system was stimulated by various observations which suggested that there were certain correlations between regulation of DNA synthesis and mitosis in eukaryotic cells and NAD metabolism. Inverse correlations between NAD content per cell, NAD pyrophosphorylase activity, and mitosis have been reported by a number of workers (*5, 6*). For example, the NAD content per *Escherichia coli* cell is inversely correlated with the growth rate. It should be noted that prokaryotic cells do not contain poly-ADPR polymerase or polymers of ADPR. It is also of interest that the NAD levels in regenerating liver cells are much lower than in nonregenerating liver cells. The same inverse correlations between NAD levels, growth, and mitosis have also been demonstrated in neonatal and regenerating liver as well as in hepatomas. Alkylating agents which induce transformation of normal cells to malignant cells also have been shown to induce NADase activity (*5*).

We have been interested in relating the ADP-ribosylation of nuclear proteins, catalyzed by poly-ADPR polymerase, with control events in DNA replication during the cell cycle of human cells in tissue culture (*1, 2*). These studies were stimulated by observations by Burzio and Koide (*7, 8*) who related poly-ADPR polymerase activ-

[1] Presented at the First Symposium of the International Chalone Conference, Brook Lodge, Augusta, Mich., June 5–7, 1972.

[2] Supported by Public Health Service grant No. CA13195 from the National Cancer Institute.

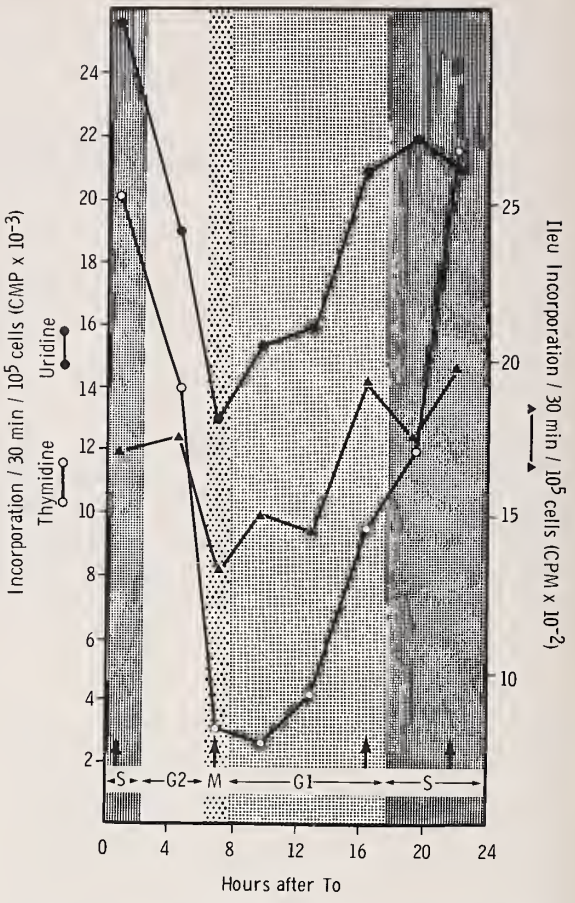
ity with DNA synthesis in vitro. These workers showed that, with isolated rat liver nuclei under conditions of maximal ADP-ribosylation of nuclear proteins, the capacity for in vitro DNA synthesis was markedly inhibited. However, during conditions such that poly-ADPR polymerase activity was inhibited in the nuclei (by end-product inhibition by nicotinamide) or such that the poly-ADPR polymer was unstable, DNA replication was not inhibited. These workers, in addition, demonstrated that chromatin which had previously been ADP-ribosylated was active in DNA synthesis only when the fraction containing ADPR was chemically removed (*8*).

These observations suggest that the ADP-ribosylation of nuclear proteins might be a normal control mechanism of the eukaryotic cell for preventing unscheduled initiation of DNA synthesis and hence prohibiting mitosis. In order to demonstrate that this theory was of significance physiologically, we investigated the activity of poly-ADPR polymerase during various stages of a synchronized human cell cycle and the relationship of enzyme activity and poly-ADPR polymer formation to general control of mitosis and growth in human cells.

MATERIALS AND METHODS

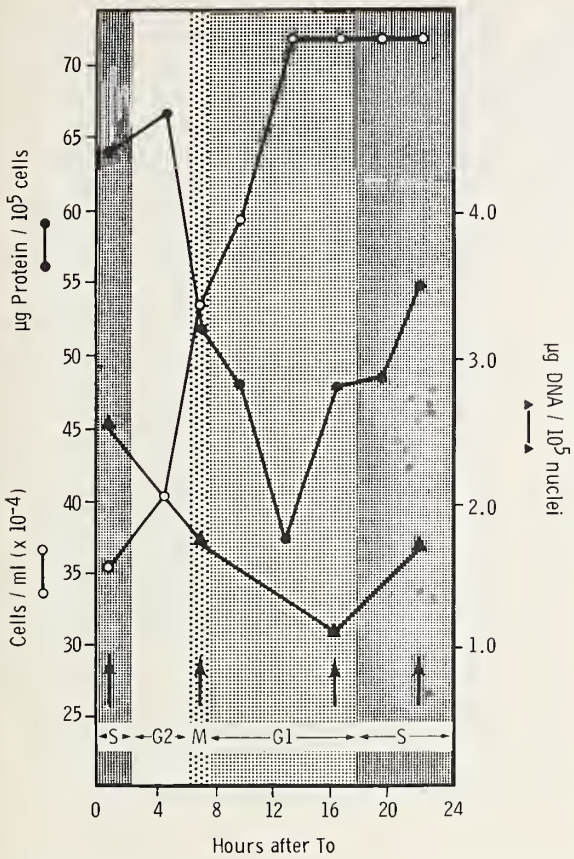
Cells.—HeLa S3 cells were synchronized by the double thymidine block method (*1*). After the second thymidine blockage, when the cells had reached the boundary between G_1 and S phases, thymidine was removed from the culture by centrifugation; the cells were allowed to proceed approximately 9 hours into S phase. Various measurements were performed to establish the characteristics of the cell cycle.

Studies of synthesis of macromolecules during the cell cycle indicated that the population of cells was finishing S phase at the onset of the experiment, as shown by the high rate of thymidine incorporation per cell (text-fig. 1). The first S phase of this experiment was separated from a second S phase, as indicated by the second wave of thymidine incorporation, by approximately 14 hours. The cell cycle in this experiment was approximately 24 hours. All macromolecule synthesis dropped to a minimum at



TEXT-FIGURE 1.—Rates of DNA, RNA, and protein synthesis in HeLa cells during cell cycle. Measurements were begun 9 hours after the second thymidine block. At various intervals, 1 ml of culture was incubated for 30 minutes with either 0.9 μCi of [CH_3-^{3}H]thymidine, 1.5 μCi of [5-^{3}H]uridine, or 2 μCi of [1-^{14}C]isoleucine. Rates of incorporation were adjusted to cell concentrations. Approximately 1.5×10^5 cells were removed for isolation of nuclei at the times indicated by *arrows*. Reproduced from (*1*) with kind permission of *Biochemical and Biophysical Research Communications*.

M phase, as reported by other workers; in addition polyribosome disaggregation occurred at M phase (not shown), also in agreement with the work of others (*9*). The DNA concentration per cell was approximately twice the G_1 level at the first S phase and approached this value in the second S phase (text-fig. 2). These fluctuations are consistent with the concept that DNA replication occurs during S phase. The content of



TEXT-FIGURE 2.—Cell growth, protein concentration, and DNA concentration of nuclei during the cell cycle. Reproduced from (*1*) with kind permission of *Biochemical and Biophysical Research Communications.*

protein per cell also followed the same general trend. Cell division occurred after mitosis.

Approximately 10^8 cells were collected at four periods during the cell cycle, as indicated by the arrows in text-figures 1 and 2—late S phase of one cell cycle, a period approximating mitosis in the cell cycle, G_1 phase, and a second S phase. Nuclei were isolated from the collected cells and purified by various centrifugal and detergent washes (*1, 2*). These nuclei were essentially free from cytoplasmic contamination as indicated by lack of cytoplasmic enzymes and by analysis of nuclear RNA content.

Enzyme assay.—As shown by other workers, the ADP-ribosylation of histones in intact nuclei is dependent upon NAD, is severely inhibited by the end product of poly-ADP-ribosylation, nicotinamide, and shows a requirement for DNA. The fluctuations in specific activity of RNA polymerase were not very great (table 1). However, the specific activity of this enzyme correlated well with the rates of RNA synthesis in the intact cells during the cell cycle as indicated in text-figure 1. For example, the rate of RNA synthesis during mitosis was very low in intact cells, and the specific activity of RNA polymerase was low at this stage of the cell cycle; the rate of RNA synthesis was high in the intact cell during G_1, and a higher specific activity of RNA polymerase was noted in nuclei at this stage.

RESULTS AND DISCUSSION

The changes in specific activity of poly-ADPR polymerase during the cell cycle were quite different from those in RNA polymerase (table 1). The specific activity was minimal during late S phase of one cell cycle and early S phase of the second cell cycle. In contrast, it was high during mitosis and G_1, periods of the cell cycle when scheduled DNA synthesis does not occur.

These results with intact human cells confirm the results Burzio and Koide obtained in in vitro experiments (*7, 8*). They suggest that the

TABLE 1.—*Specific activities of poly-ADPR polymerase and RNA polymerase of nuclei isolated during cell cycle*

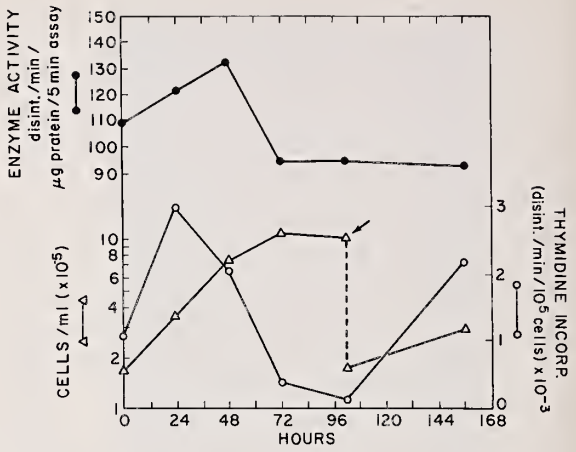
Cell phase	Hours after T_0	Rate of DNA synthesis (cpm/30 min/10^5 cells)	Poly-ADPR polymerase*		RNA polymerase* dpm UMP incorporation/ μg DNA
			dpm/μg protein	dpm/μg DNA	
Late S	0.67	20,177	4.8	82	143
M	7.00	3,046	14.5	247	105
G_1	16.25	9,645	16.7	376	166
Early S	22.00	21,701	8.5	204	115

*Each assay was performed in duplicate; the average difference was 10%.

function of poly-ADPR polymerase in nuclei might be associated with control of the initiation of DNA synthesis. Thus this histone modification might be related to suppression of the frequency of unscheduled initiation of events of DNA replication. Other modifications of histones have been studied during the cell cycle (*10, 11*). It is clear that certain types of histone modification might be related to transcriptional control (as might be ADP-ribosylation also). Phosphorylation of histones has been shown by many workers to occur maximally in the intact cell during S phase (*5, 10*). This is in contrast to poly-ADPR polymerase, whose activity was very low during S phase. However, when nuclei are incubated under conditions such that phosphorylation of histones occurs (simulating S phase events), the poly-ADPR polymerase activity is inhibited (*12*). This is what would be expected from the observed low activity of the enzyme at S phase in the intact cell.

Also consistent with our observations in intact cells (*1, 2*) and the data of Burzio and Koide (*7, 8*) are the observations of Haines et al. (*13*) who studied enzyme activity of rat liver nuclei fractionated in a zonal sucrose gradient. They could show in vitro that those nuclei which had been engaged in active DNA synthesis in the intact rat had virtually no poly-ADPR polymerase activity; however, those nuclei which had been inactive in DNA synthesis in the intact rat liver showed high poly-ADPR polymerase activity. This again indicates an inverse correlation between DNA replication and poly-ADP-ribosylation of nuclear proteins. In addition, Clark and coworkers (*5, 6*) have shown that the increase in DNA synthesis which occurs in the regenerating liver system is correlated with a decrease in NAD levels in liver and also in poly-ADPR polymerase activity.

The data thus far have indicated that a control function of poly-ADPR polymerase might be involved in preventing improper initiation of DNA synthesis in cells during normal physiological growth conditions (*1*). We also examined the activity of this enzyme during the asynchronous cycle of a population of HeLa cells (*2*). Text-figure 3 describes the normal characteristics of HeLa cell asynchronous growth



TEXT-FIGURE 3.—Relationship of poly-ADPR polymerase activity to growth and DNA synthesis in HeLa cells. At *arrow*, cell concentration was adjusted by the addition of fresh medium. Approximately 1.5×10^8 cells were removed for isolation of nuclei at each point of enzyme assay. DNA synthesis was measured by incubating 1 ml of culture with 0.9 μCi of [CH_3-^{3}H]thymidine for 10 minutes. The specific activity of poly-ADPR polymerase, when assayed for 30 minutes, followed essentially the same pattern as in the 5-minute assay. Reproduced from (*2*) with kind permission of *Biochimica et Biophysica Acta*.

—i.e., an exponential phase for approximately 24 hours followed by a late logarithmic phase which is followed by a stationary phase in which cell growth was inhibited. At approximately 96 hours, the stationary cells were diluted with fresh medium to initiate cell growth and reinitiate DNA synthesis (indicated by thymidine incorporation per cell). The maximal amount of initiation of DNA synthesis per cell occurred during midlogarithmic phase (24 hr). By late logarithmic phase, DNA synthesis was decreasing. DNA synthesis was rapidly initiated at 96 hours, when growth was stimulated.

The specific activity of poly-ADPR polymerase assayed in nuclei isolated at various times during this growth cycle is also shown in text-figure 3. The highest amount of ADP-ribosylation of nuclear proteins occurred when the cells had reached the late logarithmic or stationary phase of cell growth, when DNA synthesis was greatly diminished. Again it appears as if the activity of this nuclear enzyme is associated with

periods of physiological growth of cells when programed synthesis of DNA is to be prevented by the cell. During the latter part of this experiment, when DNA synthesis was rapidly reinitiated by cell dilution, the specific activity of poly-ADPR polymerase remained at the resting level. This indicates that there is no direct correlation between this activity and initiation of DNA synthesis. The relationship seems to be an inverse one.

We also investigated the activity of poly-ADPR polymerase during gene activation of human lymphocytes by phytohemagglutinin (PHA). Pogo et al. (*14*) have shown that acetylation of histones is one of the earliest events in the PHA stimulation mechanism, with activity appearing as early as 15 minutes after addition of the mitogen. PHA caused an immediate (24 hr) stimulation of DNA synthesis, and during this period poly-ADPR polymerase activity remained at control levels (table 2). However, the specific activity of the enzyme was increased 10-fold by 72 hours and 20-fold by 96 hours. These data are in contrast to our experiments with HeLa cells. The involvement of ADP-ribosylation of nuclear proteins in the lymphocyte-gene activation system must await further experimentation.

Since cyclic AMP has been implicated in the control of certain histone modifications as well as in the inhibition of turnover of the poly-ADPR polymer (*5, 12*), it was of interest to investigate the influence of this nucleotide on the above processes. Intact HeLa cells were incubated with dibutyryl cyclic AMP (1 mM). Preliminary data indicate that DNA synthesis is inhibited approximately 40%, RNA synthesis is unaffected, and protein synthesis is stimulated about 25%. Nuclear RNA polymerase and poly-ADPR polymerase were stimulated 56 and 30%, respectively.

HeLa cells were incubated for approximately 5 hours under various conditions which would tend to restrict various types of macromolecular synthesis selectively (*2*): amino acid deprivation, to inhibit protein synthesis and RNA synthesis (*15, 16*); cycloheximide, to inhibit protein synthesis; actinomycin D, to inhibit RNA synthesis; and hydroxyurea and cytosine arabinoside to inhibit DNA synthesis. Nuclei were isolated and purified and assays were performed for RNA polymerase as a control and for poly-ADPR polymerase. Both rate and the acceptor activity (20 min) of chromatin for the poly-ADPR polymer were measured for the latter enzyme. The activity of poly-ADPR polymerase was relatively unaffected by inhibition of protein synthesis in intact cells for as long as 5 hours (table 3). This indicates that the enzyme is not strongly degraded in the intact cell, in contrast to evidence which indicates that the poly-ADPR polymer is rapidly turning over. The two antagonists, hydroxyurea and cytosine arabinoside, which inhibited DNA synthesis in the intact cell almost completely, stimulated the initial activity of poly-ADPR polymerase in nuclei isolated from these cells. This confirms our observations that, when DNA synthesis is decreased in cells, the activity of poly-ADPR polymerase seems to be increased. Inhibition of RNA synthesis by actin-

TABLE 2.—*ADP-ribosylation of histones during gene activation in human lymphocytes**

Experimental period (hr)	Thymidine incorporation into DNA (cpm)		Poly-ADPR polymerase (cpm/mg nuclear protein)	
	Control	PHA	Control	PHA
24	458	4,585	—	105
48	—	9,371	—	—
72	498	16,965	112	1,035
96	220	31,190	113	2,374

*Normal human lymphocytes (courtesy of Dr. Graham Jamieson, American Red Cross) were incubated in the presence and absence of PHA. At the times indicated, 40-ml samples (8×10^5 cells/ml) were removed for isolation of nuclei (*1, 2*). Poly-ADPR polymerase (8-min assay) was performed as previously described (*1, 2*). DNA sythesis was followed in 4-ml samples of control and PHA-stimulated cells by 1-hour thymidine-^{3}H pulses.

TABLE 3.—*Effect of various inhibitors on poly-ADPR polymerase activity*

Additions	Synthesis in intact cells (% of control)			Poly-ADPR polymerase (dpm/μg protein)		RNA polymerase (dpm/μg protein) (% of control)
	Protein	RNA	DNA	5 minutes	20 minutes	
None*	100	100	100	4.7	11.6	100
Minus isoleucine + O-methylthreonine (6mM)	31	32	11	3.8	10.4	70
Nicotinamide (20 mM)	160	194	127	3.3	9.0	96
Cycloheximide (0.1mM)	23	94	10	3.4	10.2	75
Actinomycin D (5 μg/ml)	29	3	1.4	0.9	2.9	39
Hydroxyurea (10 mM)	157	222	1.5	7.6	11.2	97
Cytosine arabinoside (10 μM)	176	273	2.3	6.2	11.1	98

*Absolute values were: protein synthesis, 48 dpm incorporated/10^5 cells/15 minutes; RNA synthesis, 3791 dpm; DNA synthesis, 2139 dpm; RNA polymerase, 15 dpm incorporated/μg protein/30 minutes.

omycin D had a marked inhibitory effect on both the rate of the reaction and on the yield of the poly-ADPR polymer. Although it is well established that this enzyme has a strict requirement for DNA template, RNA has not been implicated in its mechanism.

Various experiments were performed to characterize the involvement of RNA with poly-ADPR polymerase activity. Control experiments indicated that actinomycin D did not affect poly-ADPR polymerase in vitro (*2*). That is, when nuclei were incubated with concentrations of actinomycin D which were sufficient to inhibit nuclear RNA polymerase by close to 90%, no effect on poly-ADPR polymerase activity was noted. This indicated that the inhibitory action of actinomycin D on this enzyme had to occur in the presence of the intact cell and suggested that a species of RNA might be required for activity of the enzyme.

The data in table 4 also support this concept. In this experiment, RNA synthesis in intact HeLa cells was inhibited by cordycepin (*3'*-adenosine). This antagonist, an adenosine analog, is capable of inhibiting synthesis of various types of nuclear RNA in intact cells by virtue of chain termination (*17*), a mechanism different from that of actinomycin D. Nuclei isolated from cells incubated with cordycepin also had decreased poly-ADPR polymerase activity. Recently it has been shown that cordycepin specifically inhibits the synthesis of the poly-adenylate residues which are attached to nuclear precursors of messenger RNA (*17*). It is tempting to speculate that there might be a relationship between the formation of the poly-ADPR polymer in nuclei and the modification of messenger RNA with poly-adenylate groups during RNA synthesis. These relationships are under study at present in our laboratory.

Other experiments (*2*) have indicated that the requirement for in vivo RNA synthesis for proper functioning of poly-ADPR polymerase is strict; inhibition of RNA synthesis for as little as 30 minutes decreases the activity of the enzyme. Actinomycin D concentrations of 1 mg/ml were required for this effect (*2*).

Whereas Burzio and Koide (*7, 8*) showed that ADP-ribosylation of nuclear proteins decreased the capacity of nuclei to carry out the DNA polymerase reaction, in preliminary experiments we have found that ADP-ribosylation of HeLa cell nuclei stimulated in vitro RNA polymerase activity. Thus it is possible that a further function of this unique histone modification might be involved in various types of transcriptional control mechanisms of the intact cell.

In conclusion, there is no evidence at this time that poly-ADPR polymerase is involved with a chalone mechanism in HeLa cells. However, there are two interesting relationships between chalones and ADP-ribosylation of proteins. Cyclic AMP has been shown to inhibit the turnover of poly-ADPR polymer in intact nuclei and hence, according to our data, to prevent unscheduled synthesis of DNA. One proposal for the mechanism of action of chalones involves the accumulation of cyclic AMP in cells (*18*). Hence, both chalones and the poly-ADPR polymer would tend to depress the abil-

TABLE 4.—*Effects of cordycepin and actinomycin D on nuclear poly-ADPR polymerase in vivo*

Experiment No.	Addition to medium	Synthesis in intact cells (% of control)			Poly-ADPR polymerase (% of control)
		Protein	RNA	DNA	
1	None*	100	100	100	100
1	Cordycepin (50 μg/ml)	87	57	78	78
1	Actinomycin D (1 μg/ml)	82	5	50	78
2	None†	—	100	—	100
2	Cordycepin (100 μg/ml)	—	56	—	70

*Absolute values were: protein synthesis, 1313 dpm incorporated/10^5 cells/10 minutes; RNA synthesis, 22,500 dpm; DNA synthesis, 2471 dpm; poly-ADPR polymerase, 151 dpm incorporated/μg nuclear protein/2 minutes.

†Absolute value: poly-ADPR polymerase, 445 dpm incorporated/μg nuclear protein/2 minutes.

ity of the cell to enter into mitosis. Also, nicotinamide has been shown to be an inhibitor of mitosis. Nicotinamide is also a precursor of NAD, which is required as a substrate for poly-ADPR polymerase.

Note added in proof: Recently we have found that ADP-ribosylation of HeLa nuclei greatly activates the template capacity of these nuclei for DNA polymerase from *Escherichia coli*. Alkaline sucrose gradient analysis indicates that the mechanism of this stimulation is an increase in nicks in the DNA. This interesting effect is most marked in G_2.

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The Melanocyte Chalone[1]

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SUMMARY—Relatively few papers have been published on the control of melanocyte division. There is no evidence for any major control mechanism other than a specific melanocyte chalone. Adrenaline and hydrocortisone may or may not be required for optimal activity. The chalone appears to be a very small glycoprotein or glycopolypeptide.—Natl Cancer Inst Monogr 38: 213–216, 1973.

MALIGNANT MELANOMA in humans is reported in England at a rate of between 2 and 3 per year per 100,000 of the population, or about 1% of all cancers. A melanoma is usually a very dark or black tumor formed by an uncontrolled growth of the pigment-forming cells of the body. When this condition is malignant, the prognosis is poor. If the condition is diagnosed before the cells have spread to remote parts of the body, surgical removal is the best treatment. Treatment by radiotherapy is in general not recommended because melanomas are frequently found to be surprisingly resistant to radiation (*1*). This fact alone makes the melanocyte different from most other cells in the body, although it has been found that melanoma cells grown in culture are no more resistant to radiation than are many other cultured mammalian cells (*2, 3*).

The melanocyte has another special place among the mammalian cell types. If all melanocytes were selectively removed from an animal, all pigment would be lost but this by itself would not be lethal. Thus any method of selectively removing melanocytes without damaging other cell types could be used as a cancer cure even in the presence of widespread metastasis. Such attempts have already been made. For example one can use the pigment-forming properties of these cells in such a way that they cause their own destruction. The drug 4-hydroxyanisole is oxidized by the tyrosinase in melanocytes to give rise to toxic free radical derivatives (*4*). This drug has already been shown to kill melanocytes selectively in a mixed culture (*5*) but unfortunately initial clinical experience with the drug has not been quite as successful as had been hoped (*6*).

A more certain way of selectively slowing or stopping the growth of melanocytes would be the injection of large amounts of a melanocyte chalone; there is already substantial evidence for the existence of such a substance. Giacometti and Allegra (*7*) have shown that after wounding there is a wave of mitosis in melanocytes among the epidermal cells in the guinea pig. The time sequence observed for melanocyte mitosis differed from that of epidermal mitosis and thus it is reasonable to assume that such cells have their own independent regulation mechanism.

At about the same time, Bullough and Laurence (*8*) showed that crude commercial pig skin extract which contained an epidermal chalone would also decrease the mitotic rate of melanotic melanoma, but only in the presence of adrenaline and hydrocortisone. However, highly purified pig skin epidermal chalone had no effect on the melanoma and thus it can be inferred that the activity of the crude extract

[1] Presented at the First Symposium of the International Chalone Conference, Brook Lodge, Augusta, Mich., June 5–7, 1972.

on melanocytes was probably not due to the action of the epidermal chalone but to some impurity in the extract. Acetone extracts obtained from hamster melanotic melanoma and from Harding-Passey mouse melanoma were also found to have this property of inhibiting mitosis of melanotic melanoma cells. These extracts were also active against the Green Fortner melanotic melanoma (*9*).

Mohr et al. (*10*) extended this work to the in vivo situation by using melanomas in hamsters and in mice. Animals bearing the tumor were given injections of partly purified pig skin chalone extract. In these experiments a dose was found which, when given daily for 5 days, resulted in complete regression of the tumors. After smaller doses, partial regression was observed; larger doses were lethal, presumably because of some toxic material in the extract.

In my laboratory we[2] have found that extracts of Harding-Passey melanoma grown in mice will inhibit the growth of the same Harding-Passey cells grown in culture. Our initial studies were based on measurements of the amount of pigment produced, on the assumption that this would be a measure of the amount of cell differentiation in the culture, but we soon found that our assumption was wrong. Kitano and Hu (*11*) have shown that the rate of pigment production is dependent on cell concentration. In our own work we confirmed this observation but found that adding a crude melanoma extract to a low concentration of cells resulted in excessive pigment production. This pigment-producing property of the extract could be easily separated from the growth-inhibiting property by heating for 15 minutes at 50° C. Also, when the extract was dialyzed the two effects were completely separated. The growth inhibitor was dialyzable but the color-promoting activity remained inside the dialysis bag. The nondialyzable fraction made the culture media turn black even in the absence of cells, so we assumed that the active ingredient in this color-promoting fraction was tyrosinase and did no more work on it.

The growth-inhibiting action of the extract is specific to melanocytes in that we have found it to have no effect on Chinese hamster lung cells in culture, on transformed human embryo liver cells in culture, and on normal human fibroblasts. These are only preliminary experiments but they give us hope that we are dealing with a melanocyte chalone. This substance differs from the majority of other chalones in that it is dialyzable and that it exerts its action in the absence of adrenaline and hydrocortisone. However, in both these properties it is similar to the granulocytic chalone extensively studied by Rytömaa and Kiviniemi (*12*) and Paukovits (*13*).

MATERIALS AND METHODS

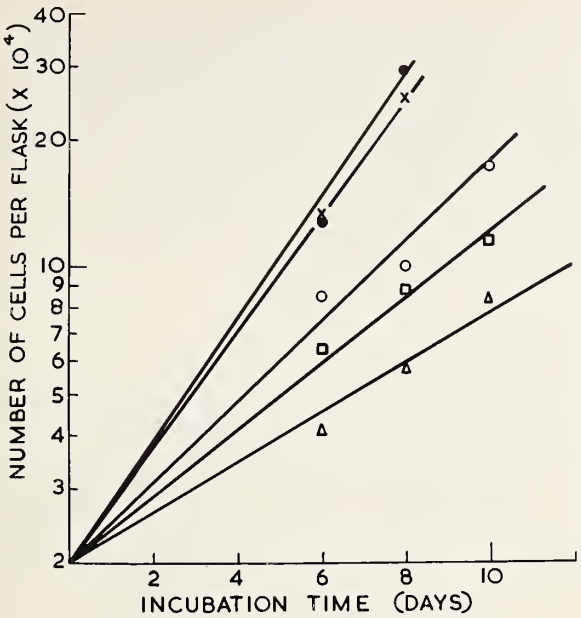
Our standard technique for measuring the activity of the extract is to add a known volume of the extract to 1–2 $\times$ 10^4 melanoma cells growing in a plastic tissue-culture flask. The flasks are then sealed to retain the correct CO_2 concentration and incubated at 37° C for 6, 8, or 10 days, after which time they are tested in the following way. The cells are removed from the surface with trypsin, pelleted by centrifugation, resuspended in 1 ml of saline, and counted in a blood cell counting chamber. Duplicate counts on a Coulter counter were found to be, on the whole, less accurate and were discontinued. Counts higher than 3 $\times$ 10^5 are disregarded because cells tend to become detached from the plastic surface when grown in high cell concentrations and this can give misleading results.

RESULTS

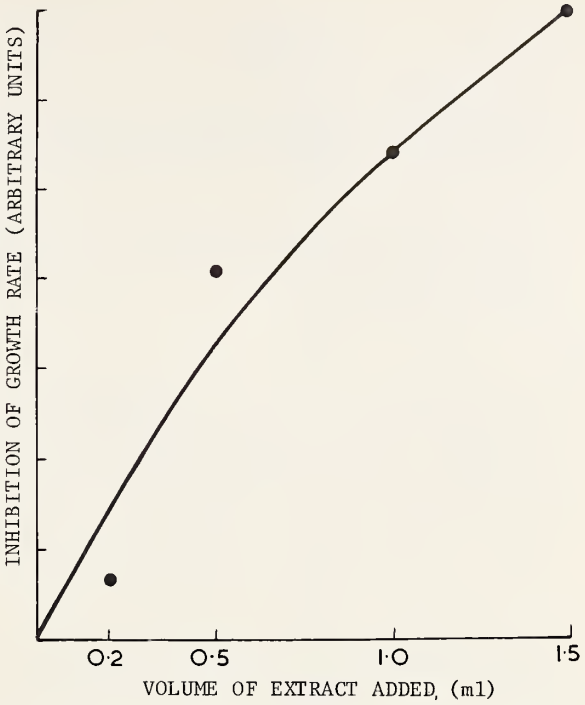
Text-figure 1 shows the results of a typical experiment and demonstrates that increasing the amount of extract decreases the growth rate of the cultured cells. In text-figure 2, the slopes of the growth rate lines from text-figure 1 are plotted against the amount of extract added, to form a dose-effect curve. Although this is not a straight line, it is not very far removed from one; we do not yet have sufficient data to be sure of the precise shape of the line.

Some preliminary observations have been made on the properties of this substance. Trypsin alone had no significant effect on the cells but trypsin incubated with the extract for 1

[2] With Dr. C. Clarkson and Miss P. Duffin.



TEXT-FIGURE 1.—Number of Harding-Passey cells in each culture flask after continuous incubation at 37° C in presence of the following amounts of dialyzed extract (ml/flask): Δ = 1.5; □ = 1.0; ○ = 0.5; × = 0.2; ● = control, no addition.



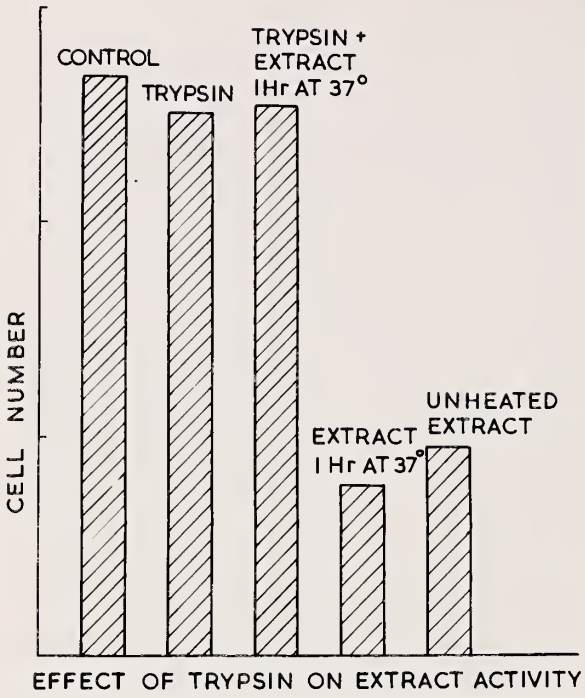
TEXT-FIGURE 2.—Slope of lines in text-figure 1 plotted against volume of extract added to each flask.

hour at 37° C completely abolished the activity (text-fig. 3). Incubation for shorter periods or at a lower temperature had proportionally less effect. Similar results have also been obtained with chymotrypsin. Thus it looks as if the active ingredient of the extract is a dialyzable polypeptide or protein containing at least one arginine or lysine residue and at least one aromatic amino acid. The amount of material available to date has been far too small for any form of chemical analysis, so we have tried to follow the biological activity after various purification procedures.

On Sephadex G-25 we obtained two fractions; one was fast-moving, indicating a molecular weight over 10,000 Daltons. This value is rather high for something which has been dialyzed unless some aggregation of smaller units has taken place. The other fraction seemed to have a molecular weight under 2,000 Daltons. Fractionation on Sephadex G-15 indicated that the molecular weight was even less than 2,000 Daltons, but the molecule was too large to be retained by Sephadex G-10. Indeed, addition of dry Sephadex G-10 to the extract has been useful in concentrating the active constituent. The anomalous behavior on Sephadex might be expected if the molecule contained *N*-acetylneuraminic acid (*14*). A recent experiment has shown that the extract is inactivated by the enzyme neuraminidase and thus we must assume that the substance contains amino sugars as well as amino acids. We have not yet measured its solubility in alcoholic solutions.

DISCUSSION

Bullough and Laurence (*8*) have shown that crude commercial pig skin extract contains a substance with the activity of a melanocyte chalone. The activity appeared in the 71–80% ethanol fraction and required the presence of adrenaline and hydrocortisone. They also reported that a 71–80% ethanol extract of a Harding-Passey melanoma was inactive, the activity in this case residing in the 80% ethanol



TEXT-FIGURE 3.—Relative number of cells per culture flask after 8 days' growth in presence of extract, trypsin, and trypsinized extract. Trypsin destroys the activity of the extract.

supernatant fraction. One explanation is that the pig skin contains some other substance which coprecipitates with the chalone between 71 and 80% ethanol. Another is that chalones produced by different species are chemically different even though they are not species-specific. The material we have extracted from the Harding-Passey melanoma is also different in that it does not require hydrocortisone and adrenaline to demonstrate its effect. This could be a species difference, but Snell (*15*) has reported that hydrocortisone administration and adrenalectomy produced no effect on the activity of the melanocytes of guinea pigs in vivo. On the whole, the differences between the various sources of material and test systems are minor in comparison with the general overall agreement.

There is substantial evidence for the existence of a melanocyte chalone. Our own work on the properties of this substance is preliminary but suggests that it is the smallest chalone yet decribed, with a molecular weight possibly less than 2,000 Daltons. The substance is quickly destroyed by temperatures over 50° C or by trypsin, chymotrypsin, and neuraminidase. We think we are examining a very small glycoprotein and we are actively looking at the nature of this molecule in greater detail.

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How Homeostasis May Be Mediated by Chalones [1]

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SUMMARY—The ways in which chalones (tissue-specific mitotic inhibitors) can work to mediate homeostasis are discussed both biologically and mathematically. Two particular systems are considered: The first, exemplified by the granulocytes, is one in which there are differentiated functional cells and undifferentiated stem cells; in the second, the tissue regulates itself. Here, liver regeneration is considered in some detail and the theory is fitted to published experimental data; an experiment is suggested and the result is predicted. Some difficulties are considered.—Natl Cancer Inst Monogr 38:217–224, 1973.

IT IS well known that many tissues in the adult animal are undergoing continual cell loss and replacement (*1*). This fact is highlighted when particular organs are partially removed: There is then a massive burst of regeneration which ceases when the organ approaches its original size. In seeking to explain these facts, two classes of theory have been put forward. The first concentrates on functional demands and suggests that organs reach a size appropriate to the body's needs [e.g., Goss (*2*)]. The second suggests that organs have a "natural" size which is dynamically maintained by regulators of growth which are continuously being synthesized and lost.

These approaches are not mutually exclusive, because biologists are still ignorant about growth regulation in most tissues. One of the few situations in which a good "story" has emerged is in the skin epidermis where it is now generally accepted that there are growth inhibitors (chalones) which regulate homeostasis (*3*). Data on other systems are ambiguous but in the liver, for example, the evidence (*4–6*) is compatible with a chalone model. The well-known cellular hypertrophy of muscle after repeated exercise is more readily comprehended in a functional framework.

It is the purpose of this paper to show how one may derive models of homeostasis in the granulocytic system and in the liver from simple, but plausible, biological premises. The central theme of these models is that the tissues regulate their own size through the manufacture and systemic distribution of tissue-specific mitotic inhibitors (chalones); these molecules regulate growth in either stem cells or the functional cells of the tissue itself. It is not claimed that the tissue uses the postulated system in vivo. It is claimed only that it could do so. Indeed, it is one of the simplest mechanisms for achieving homeostasis in these systems and one not as yet negated by any compelling evidence. It is hoped that these models will suggest experiments and predict results to deny or provide circumstantial evidence for the approach. Integral to the model are suggestions about the kinetic properties of chalones in mediating homeostasis. These may be helpful both in extraction of the molecule and in assaying its effectiveness in vitro.

The approach used here comes in essence

[1] Presented at the First Symposium of the International Chalone Conference, Brook Lodge, Augusta, Mich., June 5–7, 1972.

[2] I thank Dr. Tom Elsdale for reading the manuscript.

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from the classic paper by Weiss and Kavanau (7) who constructed a rather complex and unwieldy theory to account for both growth and growth regulation. The present paper, however, is concerned with the growth-regulating abilities of a limited class of tissue in adult organisms alone. These situations are far simpler than those considered by Weiss and Kavanau and can therefore be investigated with simpler, and hopefully more testable, postulates.

The small amount of formal mathematics is relegated to an appendix. The calculations were carried out on an IBM 360/50 computer.

THE BIOLOGICAL PREMISES

Tissues capable of achieving homeostasis through chalones are assumed to be described by the following schema.

1) Cells in the adult tissue are not permanent; a fixed proportion (P) die in a given time and other cells go into mitosis to replace them.

2) The cells produce mitotic inhibitors (chalones) at an uniform rate (Q). These are secreted into the bloodstream (volume, W, is assumed to be constant) and achieve a uniform concentration (C) in a time very much shorter than that required for cellular events to occur. It is also assumed, probably incorrectly, that this concentration is the same in both tissue and serum.

3) The chalone molecule has a half-life (R) as a result of either specific degradation or inherent instability.

4) The function of the chalone molecule is to inhibit the entry of a class of cell into the mitotic cycle. These may be either *a*) the functional cells of the chalone-producing tissue or *b*) non-chalone-producing stem cells which will divide, differentiate, and join the functional tissue where they will then produce chalones. Regardless of whether a tissue belongs to class "a" or class "b," we are totally ignorant of the inhibitory process and will describe the chalone-cell interaction by "$f(C)$," a function which is characterized by a decreasing value with increasing "C." If N be the number of chalone-producing cells,

$$\frac{\partial C}{\partial t} = \frac{Q \cdot N}{\text{tissue volume} + W} - R \cdot C.$$

The tissue kinetics are given for class "a" by:

$$\frac{\partial N}{\partial t} = f(C) \cdot N - P \cdot N$$

and for class "b" by:

$$\frac{\partial N}{\partial t} = f(C) \cdot S - P \cdot N$$

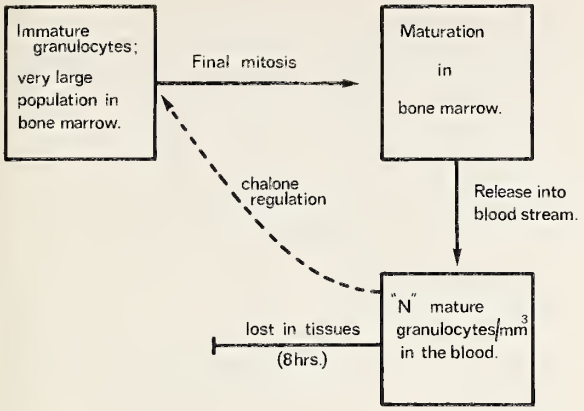
in which "S" is the stem cell population (which, for simplicity, will be considered to remain constant). Although these equations seem to have many adjustable parameters, "N_0" and "P" are found experimentally ("N_0" is the value of "N" at equilibrium). Defining the chalone variables "$f(C)$" and "R" is enough for the complete determination of the system.

The behavior of these equations near equilibrium is considered in the "Mathematical Appendix"; it can be seen that stability can be expected under biological conditions. Stable equilibrium means that, if the status quo be altered by changing the values of "N" or "C," the system will return to normal. It may do this either directly or in an oscillating manner. It can be seen from the "Mathematical Appendix" that the condition for oscillating return is that "P" is similar to or greater than "R."

This mathematical description of the biological premises should provide at least a good qualitative description of a tissue whose growth regulation is chalone-mediated. The object of the exercise is to show that one can suggest reasonable chalone properties which can be fed into the equations so that the solutions describe the dynamics of the organ or tissue in vivo. While this can be dismissed as curve-fitting, the criticism is not valid: Advocates of chalone mediation are obliged to show that chalones can work. In addition, the equations, once set up, should suggest experiments with predicted results and can thus provide validity tests. Failure to find a reasonable quantitative fit between theory and experiment invalidates the premises.

THE GRANULOCYTIC SYSTEM

As an example of a case in which it is plausible to suggest that a differentiated cell system controls the mitosis of immature stem cells, we consider the granulocytes in the blood and mye-



TEXT-FIGURE 1.—Model of granulocyte system.

locytes in the bone marrow. There is a continued turnover of leukocytes from the blood (7–8 hr). The loss is replaced by mature marrow cells, and there is some evidence of feedback of the granulocytes to the immature cells to regulate their mitosis and maturation. The result is that, in normal human blood, the granulocyte count (of free circulating rather than marginated cells) stays in the region $4–10\times10^3$ cells/mm³. Under pathological conditions, this value may increase or decrease. Rytömaa and Kiviniemi (*8, 9*) have shown that in the blood there are chalone and antichalone fractions which, in vitro at least, affect mitotic rates in the marrow cells—the former decreasing and the latter increasing expected results. In showing how feedback works, variation in only the chalone concentration will be considered.

The model of the granulocytic system that is considered here is shown in text-figure 1. Immature cells in the marrow divide and form a pool, "*S*" (which will be considered to remain essentially constant for reasons outside the frame of reference). Cells in the pool go through a final mitosis under regulation by the chalone, mature, and then move, presumably under population pressure, to the bloodstream. The cells now produce chalone until they are lost in the body tissues some 8 hours later.

Under these circumstances, the equations become

$$\frac{\partial N}{\partial t} = S \cdot f(C) - P \cdot N \qquad [1]$$

and

$$\frac{\partial C}{\partial t} = Q \cdot N - R \cdot C. \qquad [2]$$

Because so little is known of the kinetics in vivo, we arbitrarily set "R" = 24 hours (chalone half-life), $S \cdot f(C) = L/(1+C)$, $C_0 = 15$, and $N_0 = 5{,}000$ cells/mm³. Under these circumstances, the equations reduce to

$$\frac{\partial N}{\partial t} \quad \frac{8 \cdot 10^4}{1 + C} - N \qquad [3]$$

and

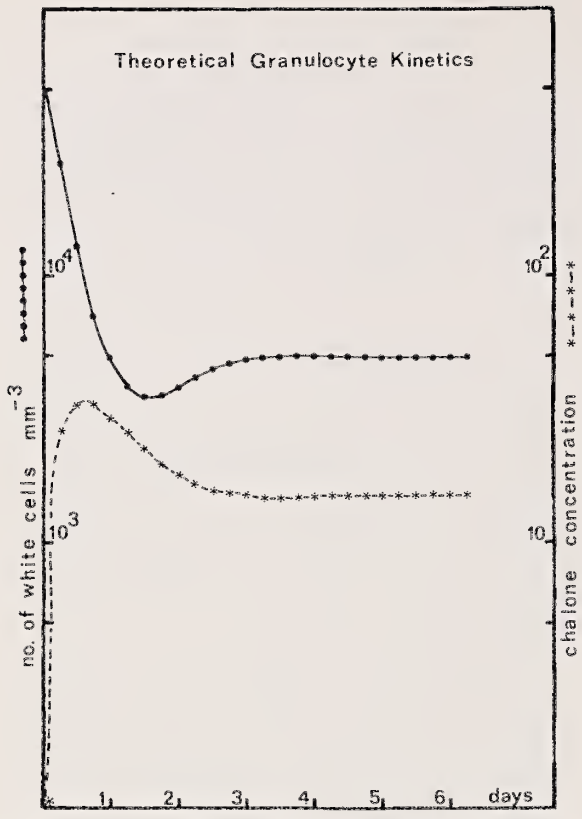
$$\frac{\partial C}{\partial t} = 3.75 \cdot 10^{-4} \cdot N - C/3 \qquad [4]$$

in which the time interval is 8 hours. Should the situation in vivo be abnormal—e.g., there are 50,000 cells/mm³ and the chalone concentration is 1—text-figure 2 shows the dynamics of the damped oscillatory return to equilibrium over a few days. It can thus be seen that this format will predict a stable homeostatic system for granulocytes in the blood.

It is emphasized that this should be considered only as the mode, not the quantitative description, of how the granulocyte chalone would work. The presence of an antichalone naturally makes the situation more complex and involves the need for a third equation. The absence of detailed data on the system means that there is not enough information to ascribe unambiguous properties to the chalones. Fortunately, the liver is more amenable to investigation.

LIVER REGENERATION IN THE RAT

The regenerative abilities of the rat liver have been succinctly reviewed by Bucher (*10*) and by Poole (*11*). In essence, the mitotic rate in a normal adult (~300 g) rat liver (~10 g) is about 0.02%, and mitosis lasts about 1 hour. Ablation of the two large lobes (removal of about 68% of the weight) results in a massive increase in DNA synthesis in the remaining lobes some 16–20 hours later, followed in 6–8 hours by a burst of mitosis with observable rates of about 4%. There is rapid increase of liver mass in the first 3 days and, within 10–15 days, the total liver mass is restored to near its former weight. This summary conceals the many biochemical and other changes that accompany regeneration and also assumes that all liver cells



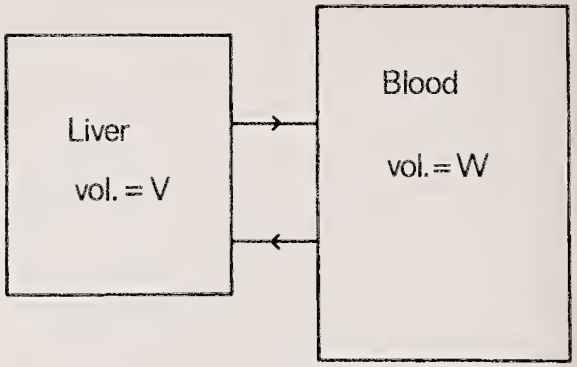
TEXT-FIGURE 2.—Theoretical granulocyte kinetics: Oscillatory return to equilibrium after a very high leukocyte count and a low chalone concentration.

behave similarly. This is not so: The parenchymal cells regenerate rapidly whereas cells in connective tissue do not. Since the parenchymal cells comprise over 90% of the liver volume, it will be assumed that they both produce chalone and regenerate—i.e., there is no liver stem cell.

The speed and size of the regenerative response (a 200-fold increase in mitotic rate) suggest that, if liver cells make self-inhibiting chalone, the behavior of the molecule must be most nonlinear with concentration. This means that relatively small changes in chalone concentration affect mitotic rates drastically. In the spirit of this heuristic approach, we let

$$f(C) = L \cdot exp(-C/C')$$

in which "C" is measured in units such that "C" = 1 and L is a constant. The equations describ-



TEXT-FIGURE 3.—Model of liver-blood system.

ing liver dynamics (using liver volume, "V," rather than cell number for convenience) become

$$\frac{\partial V}{\partial t}[L \cdot exp(-C) - P]V \quad [5]$$

and

$$\frac{\partial C}{\partial t} = \frac{Q \cdot V}{V+W} - R \cdot C. \quad [6]$$

Experimentally, $P = 0.0002$ hr^{-1}, $V_o = 10$ ml, and $W = 20$ ml (text-fig. 3). If, in addition, we let $C_o = 9.5$ and the chalone half-life (R) = 1 hour, than equations 5 and 6 become:

$$\frac{\partial V}{\partial t} = [2.6\ exp(-C) - 0.0002]\ V \quad [7]$$

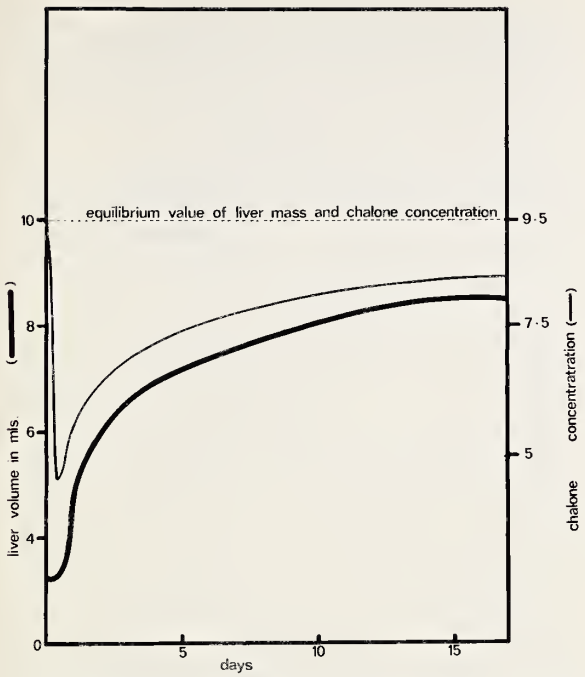
and

$$\frac{\partial C}{\partial t} = \frac{28.5V}{20+V} - C \quad [8]$$

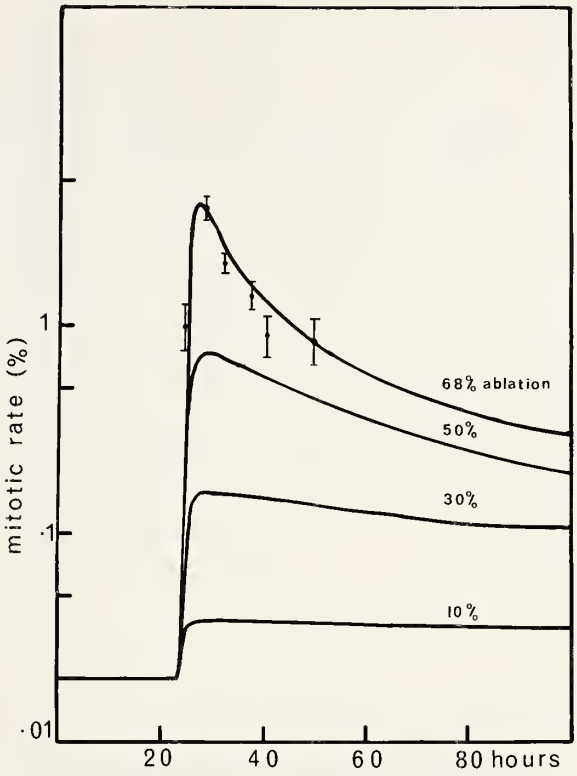
in which the time interval is 1 hour.

The stable equilibrium in liver volume is due to the use of chalone concentration rather than chalone amount. This results from using the blood volume as a chalone reservoir. If this is not so (equivalent to setting $W = 0$), it is clear that the chalone equation is independent of liver volume; there would then be no feedback between chalone concentration and liver volume and no resultant homeostasis.

Text-figure 4 shows the predicted solutions to the equations after removal of 68% of the liver and assuming (in the spirit of "The Merchant of Venice") no loss of blood. After the ablation, chalone concentrations decrease rapidly and then increase pari passu with liver volume which increases from 3.2 to 7 g in about 3 days and then grows more slowly, reaching 8.5 g in



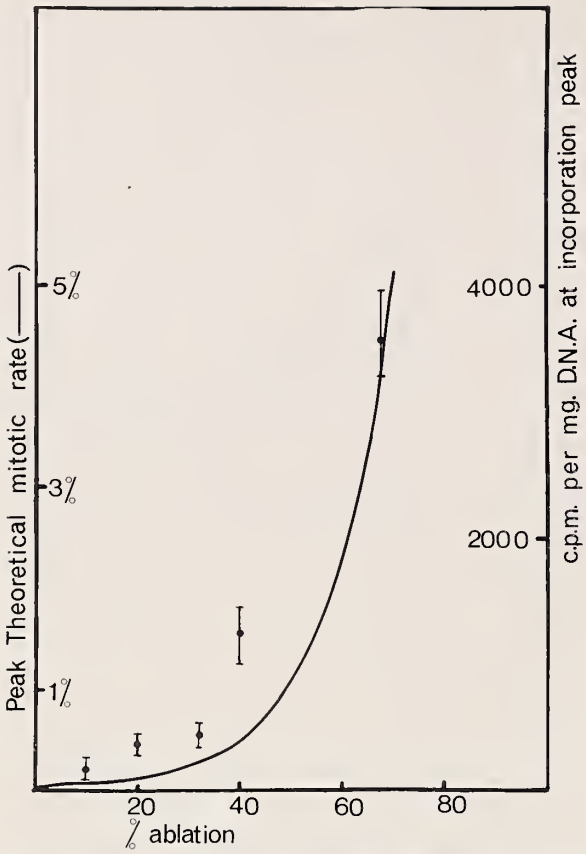
TEXT-FIGURE 4.—Theoretical liver kinetics: The change in liver volume and chalone concentration with time after 68% ablation.



TEXT-FIGURE 5.—Theoretical mitotic rates in liver after various degrees of ablation (log plot). In fitting the curves to the shown data of Weinbren (*4*), a lag of 22 hours between chalone concentration and mitosis has been inserted. Chalone half-life postulated to be 1 hour, and the degree of inhibition varies exponentially with the chalonic concentration.

about 15 days. Text-figure 5 shows the predicted mitotic rates after various degrees of ablation (a 22-hr lag between chalone concentration and mitosis is given). The experimental data of Weinbren (*4*) are also shown, and it can be seen that there is acceptable agreement. Text-figure 6 shows a comparison between predicted maximal mitotic rates after increasing degrees of ablation and the experimental data of Bucher (*10*) who measured thymidine incorporation rates. Although these are only approximately comparable, it can be seen that theory gives a qualitative fit to the form of the experimental data.

In formulating a theoretical fit to the experimental data we assign these properties: the time between cell-chalone interaction and mitosis, the biochemical kinetics of the interaction, and the chalone half-life in vivo. In order to have any reasonable fit, it turns out that a half-life of between 1 and 2 hours is needed. Short of extraction of the chalone, there seems to be little direct way of testing this suggestion. A simple indirect experiment may be possible to investigate the dose-response curve. If the chalone is degraded in the blood, storage of blood in vitro at body temperature should, after 12 hours or so, result in a marked decrease in chalone concentration. Therefore, if two immunologically identical rats are taken and various amounts of the blood of the first are replaced by stored blood of the second, there should be a temporary decrease in the chalone concentration which should at some time later result in a small burst of mitosis in the liver. This time interval should be the first parameter discussed above. The size of the response as a function of transferred volume should give insight into the kinetic interaction. It is a prediction of the model given here



TEXT-FIGURE 6.—Predicted maximal mitotic rate after various degrees of ablation is compared with peak value of thymidine incorporation after ablation as obtained by Bucher (*10*).

that there should be a response which varies exponentially with the volume transfused.

DISCUSSION

The formal treatment of tissue homeostasis given here is not meant to provide an exact description of all the observations that have been made. The contention is that a relatively simple framework exists which will account for kinetic data in certain tissues and within which other facts may be accommodated. In this paper, only the liver has been considered in detail because it is one of the few organs for which there is good kinetic information. More sophisticated, additional assumptions and procedures would certainly permit a more exact match of the theoretical prediction with the liver data but would be out of the spirit of the enterprise. The acceptable fit of what is really a very simple model is an encouraging pointer that chalones do regulate liver homeostasis in vivo.

There is an unsatisfactory aspect which should be mentioned: In matching fact and theory, it is necessary to ascribe properties to the liver chalone about its half-life and mode of action in vivo. The former is no problem—turnover is an accepted part of any biological system. But the nonlinear, exponential kinetics required for the liver is far less common. No example is known to the author; but even if none be known, it does not mean that it cannot happen. It has been tacitly assumed that chalone interacts directly with the genome to affect the probability that a cell enters the mitotic chreod. If, however, the pathway of connection is complex, then the nonlinear interaction may be understood as the cooperative kinetics of several molecules. [The problem was discussed by Walter et al. (*12*).] The question can be cleared up only by studying in detail the interaction between cell and chalone (and thus in turn will provide appropriate assay systems for further investigations); theory cannot be helpful on this point.

The framework given here should be applicable to other systems in the body. Whether the framework is a true description will only be answered by experiment. This will be difficult: Studies in vitro will be open to the normal criticisms and, in addition, the fact that a molecule can inhibit mitosis in vitro does not imply that it mediates homeostasis in vivo. Studies on animals are ambiguous because, in attempting to differentiate between "self-regulation" and "functional need," advocates of the latter approach can always claim that there are as yet unknown needs of which we are ignorant. The approach that I favor is to select, if possible, a theory that permits the facts to be correlated and to put faith in it until it is negated or a more accurate one is found.

MATHEMATICAL APPENDIX

Here, the behavior of equations of the form

$$\frac{\partial X}{\partial t} = U(X,Y) \quad \frac{\partial Y}{\partial t} = V(X,Y)$$

is considered near an equilibrium point (X_o, Y_o) where

$$\frac{\partial X}{\partial t} = \frac{\partial Y}{\partial t} = 0.$$

Near this equilibrium point (where $X = X_o + x$, $Y = Y_o + y$) it is usually possible to make the linearity assumption

$$\frac{\partial x}{\partial t} + ax + by + \varepsilon_1(x,y)$$

$$\frac{\partial y}{\partial t} = cx + dy + \varepsilon_2(x,y)$$

where ε_1 ε_2 are negligibly small. These equations have solutions of the form

$$x,y = r(t)\ exp(s\ t)$$

where s is given by the roots of the determinant

$$\begin{vmatrix} a-s & b \\ c & d-s \end{vmatrix} = 0$$

thus

$$S = \tfrac{1}{2}\left[(a+d) \pm \sqrt{(a-d)^2 + 4bc}\right]$$

$s = f \pm (g + hi)$ where g or $h = 0$. The behavior of the solution near equilibrium is given only by the exponent. Thus, if

$$f \pm g > 0 \text{ and } h = 0 \text{ or } f > 0 \text{ and } h \neq 0$$

the function increases with increasing time and thus goes away from the equilibrium point—this implies that the equilibrium is unstable (text-fig. 7, *left*). If however

$$f \pm g < 0$$

then the solution decreases with increasing time and the equilibrium is stable. When

$$h = 0$$

then the approach to equilibrium is direct (text-fig. 7, *center*) but if

$$h \neq 0 \quad g = 0$$

there is an oscillatory approach to the equilibrium point (text-fig. 7, *right*).

The criterion for homeostasis therefore is that

$$f \pm g < 0.$$

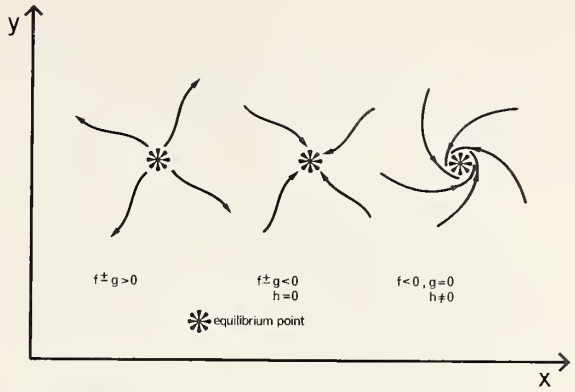
Further details may be found in any mathematical text on differential equations, e.g., Sanchez (*13*).

The Granulocyte System

Here (the case $n=1$ is considered)

$$\frac{\partial N}{\partial t} = -PN + \frac{L}{(1+C)^n}$$

$$\frac{\partial C}{\partial t} = QN - RC.$$



TEXT-FIGURE 7.—Theoretical trajectories in an area of equilibrium. *Left*, unstable; *center*, stable with direct return to equilibrium; and *right*, stable with damped oscillatory return to equilibrium.

One can readily show that

$$a = \frac{-NL}{(1+C_o)^{n+1}} \qquad b = -p$$

$$c = Q \qquad d = -c$$

Here, $b < 0$ and both a and $d < 0$; there is therefore always a stable equilibrium. The condition for an oscillatory approach to it is that

$$R - \frac{N \cdot L}{(1+C_o)^{n+1}} < 4 \cdot P \cdot Q.$$

The Liver Model

Here

$$\frac{\partial V}{\partial t} = (Le^{-C} - P)V$$

$$\frac{\partial C}{\partial t} = Q \cdot V - R \cdot C.$$

One can show, with but little trouble, that

$$a = (L \cdot e^{-C_o} - P) \qquad b = -V_o L e^{-C_o}$$

$$c = \frac{Q \cdot W}{(V_o + W)^2} \qquad d = -R$$

Here, $b < 0$ and the condition for a stable equilibrium is that

$$(L \cdot e^{-C_o} - P) - R < 0.$$

In the liver, $P = 0.0002$ hr^{-1} and, at equilibrium $_{L}e^{-C_o} = P$. Thus, provided $R > 0.0002$ (or the chalone half-life is much less than 5,000 hr), equilibrium will always be stable. The smallness of P and the rapidity of the responses in vivo (which means R is large) imply that there can never be an oscillatory approach to equilibrium.

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Summary [1]

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FOR MANY years there has been general agreement that in higher organisms there are many control mechanisms, from the molecular level up to the total organization of the body, which are controlled by the central nervous system. There is no reason that growth, differentiation, and cell loss should not also be controlled by means of similar mechanisms.

In many ways the model put forward by Weiss and Kavanau (*1*) opened up a new field and became the starting point for the chalone concept. These authors postulated that in each tissue there is a "generative mass" and a "differentiated mass." By cell division and subsequent differentiation, the generative mass feeds the differentiated mass with cells, and from the differentiated mass there is loss by virtue of cell death and catabolism. A chemical signal substance is produced by the differentiating cells and regulates the rate of cell proliferation and differentiation.

Today, great interest seems to be focused on *disturbed* growth control. To me, however, the astonishing fact is that the organs and tissues of our body are kept at a normal size. Why does the liver in a rat start to grow after hepatectomy and then stop growing as soon as it has attained about the normal size? Why is the world not filled up with epidermis? Mine just covers my body and there it stops. A beautifully regulated equilibrium between cell loss and cell renewal is constantly maintained. The most reasonable explanation seems to be that some sort of inhibiting information from the mature cells diffuses back to the dividing cells, and maybe also to the process of cell differentiation, to control and coordinate these functions with the needs of each organ and the role it plays in the whole body. Such systems are well known in hormone regulation. Therefore, from general biological principles, the chalone concept of growth regulation is easy to accept. It should be emphasized, however, that those of us who are chalone believers never think that growth control is restricted to chalone alone. A chalone is tissue-specific and therefore a very important part of the growth-regulatory system, but there are many other influences. There may be antichalones. There are growth hormones and other controlling influences from nerves and vessels. There are requirements for nutrition, mechanical pressure, and probably specific forms of contact between cells. Many such influences play different roles in the different tissues, but there is good reason to believe that chalones play first violin in the symphony orchestra of growth regulation.

Dr. Simnett discussed whether chalones are really diffusible, and thus washed away by the bloodstream, or whether there is an "antichalone" principle present. The available evidence seems to point to the fact that chalones are short-lived, but this may be due either to quick removal or to quick inactivation or binding. This seems to be a new and interesting line of research, but there is at present no evidence either for or against either of these two possibilities. Chalone-like substances have occasionally been found in the blood, and even in urine,

[1] Presented at the First Symposium of the International Chalone Conference, Brook Lodge, Augusta, Mich., June 5–7, 1972.

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but this does not exclude the possibility that rapid binding to cell constituents or degrading by antichalone substances is the main cause of the short-livedness of the chalones.

In this connection it seems appropriate to mention Dame Honor Fell's commentary from the first day's afternoon session. Her idea is that lysosomal enzymes may act as antichalones and that chalones are broken down by such enzymes. She suggested the use of lysosomal enzyme research to study the buildup and the breakdown of chalones in the tissues.

Dr. Laurence gave the history of the discovery of epidermal chalone together with an account of the various extraction and isolation procedures used. She also discussed the action of epidermal chalone on cell division in normal epithelium, both in vitro and in vivo, and in experimental pathological conditions. There is still considerable discrepancy between the results from in vitro experiments and from those performed in vivo. There is also the possibility that the various extracts from different laboratories are not identical. We need a good, reliable, discriminative test which does not rely on negative results. Only complete knowledge of the chemical composition and structure of the chalone will finally be satisfactory.

Dr. Voorhees discussed the role of cyclic AMP in the control of epidermal cell growth and differentiation. His work was based on the hypothesis of disturbed control of cell proliferation in psoriasis. In his opinion the intracellular levels of cyclic AMP are not under primary hormone control but are controlled by a specific local substance called "chalone." This paper opened up a number of interesting theories and prospects for further research on the possible interaction between cyclic AMP and chalones.

Dr. Laurence then discussed the relationship between epidermal chalone and the stress hormones, epinephrine and hydrocortisone. For many years it was believed that epinephrine was necessary for a proper chalone effect. This seems to be true only in vitro, and even there, the so-called "epinephrine wash" technique is not as discriminating as was expected. Dr. Laurence propounded the theory that chalones are primarily promoters of differentiation and that their effect on proliferation is a by-product of this. Interesting evidence, interpreted as showing a possible antichalone effect, was also put forward; and she discussed the different reactions to injections of chalone of epithelia from different body sites. I think we all agree with her conclusion that there is today enough speculation about chalones to keep experienced biologists, pathologists, and biochemists busy for a long time.

Dr. Elgjo reported work that showed that what is called the "epidermal chalone" probably consists of two different inhibitors, one acting in the late G_1 phase and the other in the late G_2 phase. The latter is probably the factor that was originally called a chalone by Bullough, because this is the factor that is measured by the Colcemid technique, while the G_1 inhibitor acts at the phase of the cell cycle in which physiological inhibitors are supposed to exert their effect, the late G_1 phase.

Dr. Marks reported experiments attempting to characterize the epidermal G_1 factor. He concluded from his extraction and purification procedures that the epidermal G_1 inhibitor is probably a macromolecule with an apparent molecular weight of more that 10^5 Daltons and is resistant to temperature, proteolytic enzymes, and other denaturing agents. He therefore suggested that this G_1 inhibitor is not identical with the classical epidermal chalone (G_2 inhibitor) as previously stated by Bullough. His results therefore fitted well with Dr. Elgjo's report.

The next morning started with Dr. Lajtha's review of leukocytes and their proliferation kinetics, focusing attention on this cell line with its rather sophisticated system of cell renewal. Thereafter, Dr. Houck presented evidence for the existence of a specific chalone for lymphocytes, supported by interesting results from Dr. Garcia-Giralt and Dr. Chung. Dr. Kiger reported similar results from Professor Mathé's group in Paris. The most interesting problem here was the use of lymphocytic chalone to prolong graft survival by suppressing lymphocytic proliferation. Personally, I feel that this is a very interesting opening for the possible clinical use of the chalones. If specific T-lymphocytic and B-lymphocytic chalones can be produced, it

might be possible to prevent immunological reactions in each of these two systems.

Dr. Rytömaa gave a survey of the granulocytic chalone. In adition to evidence presented earlier, he added some results on cell-line specificity obtained by the use of diffusion chambers implanted intraperitoneally in mice. There seems to be no doubt about the existence of a granulocytic chalone. Its purification was reported by Dr. Paukovits from Vienna. We all seem to agree that the chalone substances produced by granulocytic cells inhibit DNA synthesis of the progenitor cells of the neutrophilic granulocytes, both normal and leukemic. There is also evidence that this inhibition is cell-line specific but not species-specific and that the inhibition of DNA synthesis is short term and reversible. The molecular weight is about 4000 Daltons.

The third day we heard evidence for the existence of a fibroblast chalone, by Dr. Houck. This chalone has a molecular weight of 30,000–50,000 Daltons and an isoelectrical point between pH 3.5 and 5.3. Liver chalones were discussed by Dr. Verly and Dr. Stack-Dunne. They seem to have a molecular weight of 2000–5000 Daltons, thus resembling the granulocytic chalone. Dr. Chopra discussed possible lung and kidney chalones; and finally Dr. Bichel showed that chalone-like substances could also be found in Ehrlich ascites tumors. Here both G_1 and G_2 chalones seem to be present. The G_1 chalone has a molecular weight of 1000–10,000 Daltons; the G_2 chalone is 10,000–50,000 Daltons.

Dr. Dewey discussed the melanocytic chalone, a glycoprotein or glycopeptide which is dialyzable and has a molecular weight of about 2000 Daltons.

Dr. Bard discussed a simple model of chalone homeostasis in mathematical terms, and we heard a very interesting and sophisticated paper relating to other molecular mechanisms for control of cell division by Dr. Smulson.

What stage are we at in chalone research? My impression, after this meeting, is that we have certainly passed through infancy and are now at about the teenage level. A few years ago the mention of the word "chalone" elicited only a faint smile and a raising of the eyebrows, but today we can say it without shame. People have stopped arguing about the concept and have started discussing details, which is a good sign of the change in attitude. As mentioned by Professor Bullough, extraction and measurement of epidermal chalone have already been introduced as an undergraduate exercise for biology students at the University of Sussex. In all new pathology textbooks the word "chalone" is included in the chapter on general pathology. In immunology, complement was once a mysterious influence. When I learnt it during my medical studies, it was just an influence present in serum, and we knew some of its effects. Today we have complements 1, 2, 3, 4, etc., and we know the composition and mechanisms of action. It is my firm belief that we will also reach that level in chalone research, and that instead of "a mysterious influence in water extract of cells" we shall speak of the different well-defined chalones for the different tissues.

The danger today is of course that chalones will become too respectable and too popular. A lot of general cytotoxic effects will be called chalone effects, and there will be a lot of dust thrown up around the concept. We can only echo the hope expressed in the Arabian proverb: "When the dust falleth, Thou wilst see whether Thou ridest a horse or an ass!"

It still seems too early to formulate a final definition of the chalone. Provisionally we should reserve the word "chalone" for the overall concept of growth autoregulation by chemical signal substances. Summarizing, however, what we have agreed upon, I would say that chalones are *chemical signal substances,* they are *water soluble,* and they are *present in and produced in the tissues on which they work.* Chalones are *tissue-specific,* but *species-unspecific.* They *regulate cellular proliferation,* and maybe also the speed of *cell maturation,* but not its quality. They act in late G_1 to prevent cells from entering the S phase, and in late G_2 to regulate mitotic rate. The relation between the stress hormones and chalone is less clear today than it seemed to be some years ago. There may be a connection between chalones and cyclic AMP. Chalones are *short-lived,* but we do not know whether this is because of a chemical in-

stability of the chalone molecule itself, or because the chalones are quickly washed away by the bloodstream or because there exist specific chalone-degrading (lysosomal?) enzymes which might be called "antichalones."

One may ask why we have concentrated on looking at DNA synthesis and mitosis only. What matters for proliferation is the speed at which the cells pass through the whole cycle, and this rate can be regulated at many other stages. There is a story about a drunken man crawling about on the pavement in the middle of the night under a street light. A policeman came up and asked him what he was doing. The man said he had lost a coin, and the policeman replied: "I can help you to find it, but are you sure you lost it here?" The man explained, "I don't know where I lost it, but this is the only place with light!" And of course we must admit that we have been trained to use the stathmokinetic method to measure mitotic rate and labeled precursors to measure DNA synthesis. These methods are like two street lights that make is possible to investigate the speed at which cells pass in and out of the DNA synthesis and the mitotic compartment, and this is why we define chalones as substances that act at these definite stages of the cell cycle. It may be possible in the future, as Professor Bullough also remarked, to find other places in the cell cycle to measure. It seems biologically reasonable, however, that growth control—if it is phase-specific—should act just before S and M.

Concerning the general mechanism of chalone effect, the rate of cell division may not be directly proportional to the momentary concentration of chalones in or between the cells. Such a mechanism might be called *proportional* feedback. It may be, however, that it is not the actual concentration of chalone but the speed and the direction of alterations in concentration that are signals to which the generative mass reacts. This principle could be called *derivative* feedback. It may also be that the cells have a sort of memory: They "remember" the chalone concentrations during the previous hours and react to this accumulation. This could then be called *integral* feedback. The final result of changing concentrations of chalone on proliferation would be different in the three different situations. This needs to be investigated in more detail.

There are many more unsolved than resolved problems in chalone research. One most self-explanatory aim is to try to find a completely purified and chemically characterized chalone. This would certainly open up new possibilities for research into other chalones as well.

We have little knowledge about the mechanism of action and the possible cooperation between chalones and stress hormones and between chalones and cyclic AMP. We do not know whether chalones exert their effect *in* the cells or *between* the cells (in the cell membranes). There are many cell systems still to be investigated for the existence of possible chalones. We do not know whether all chalones belong to the same family of substances from the point of view of their chemical constitution. It seems reasonable to suppose that there might be a general proliferation-inhibitor molecule that is bound to a sort of protein or glycoprotein. The general proliferation inhibitor may be the same for all tissues, and the protein or glycoprotein part of the molecule may give each family of chalones their tissue specificity.

The most reasonable theory is that chalones are produced as a part of or in connection with cell differentiation, but we have no direct evidence for this speculation.

We also need more knowledge about the mechanism of action with regard to the cell cycle: whether chalones only block cells in late G_1 and in late G_2, or whether they can influence the cell cycle at other places.

There is certainly a need for quick and reliable test methods for each family of cells. Tissue culture has not been very profitable, since cells do not behave in the same manner in tissue culture as they do in the body. All the methods hitherto used to test chalone effects are very time-consuming.

When we have defined the chalones, we need good sources for chalone production from the different cell families. Since chalones are species-unspecific, there might be large sources of such biological substances in the larger animals.

Let us now summarize what is known about

the different chalones. It must be emphasized that some of the evidence in this field is fairly well founded, whereas other statements are without a firm scientific basis and have only been presented as possibilities based on a few series of experiments that are not absolutely conclusive.

For *the skin* there seem to be at least two chalones for the epidermis proper. The so-called *M factor,* which acts on the cells in late G_2 and which seems to be produced mainly in the basal cells, has a molecular weight of 30,000–40,000 Daltons and is probably a glycoprotein with an isoelectric point between 5.2 and 6.8. It is sensitive to heat and is unstable at physiological pH but stable at low pH. It is resistant to pepsin but is easily destroyed by trypsin. The *S factor* has a molecular weight of more than 100,000 Daltons, and is resistant to trypsin, pronase, RNase, heat, and to other protein-denaturing agents. There are indications that *sebaceous glands* and *hair follicles* of the skin have their own chalones, but these have never been purified or in any way characterized.

The *melanocyte* chalone has been partly purified and seems to be the smallest chalone yet described, with a molecular weight of possibly less than 2000 Daltons. This substance is quickly destroyed by heat, trypsin, chymotrypsin, and neuraminidase. The indications are that we are dealing with a very small glycoprotein or glycopeptide. The effect of the melanocyte chalone has been tested on the increase in number of cells of melanocytes in culture, and we do not know where in the cell cycle it exerts its effect.

Chalones inhibiting DNA synthesis in *blood and bone marrow cells* have been found for the erythrocytic, the granulocytic, and the lymphocytic series.

The *granulocytic chalone* is produced by the granulocytic cells and inhibits the DNA synthesis of both normal and malignant granulocytic cells in a short-term and reversible manner. The molecular weight is 4000–5000 Daltons, and it is possibly a small glycoprotein. Granulocytic chalone produced from bovine blood has been used for the treatment of a granulocytic chloroleukemia in rats, with very interesting results.

The *erythrocytic chalone* seems to have composition and molecular weight similar to those of the granulocytic chalone.

The *lymphocytic chalone* also seems to be a glycoprotein but has a molecular weight of about 45,000 Daltons. Interesting results have been obtained utilizing this chalone as an immunosuppressive agent.

As regards the *liver,* the situation is complicated. The literature on substances influencing the growth rate of regenerating liver after partial hepatectomy is voluminous and sometimes difficult to interpret. However, there seems to be a liver chalone of a molecular weight of 2000–5000 Daltons, probably a glycoprotein.

There are indications of chalone-like substances in the *kidney,* but difficulties in interpretation of the results are similar to those described for the liver.

The *fibroblast chalone* has recently been partly characterized. It seems to have a molecular weight range of 30,000–50,000 Daltons, and it has been measured by determining the rate of entrance of cells into DNA synthesis. The substance is destroyed by heat and trypsin digestion, and it has an isoelectric point somewhere between 3.5 and 4.3. It is also probably a glycoprotein.

There are some indications of chalones in *lung alveolar epithelium* and some information on chalones in the *lens of the eye.*

Chalone-like substances have been found in *ascites tumor cells.* Both G_1 chalone (1000–10,000 Daltons) and G_2 chalone (10,000–50,000 Daltons) have been found. Specific chalones are indicated also in *leukemic cells, melanomas,* and *squamous cell carcinomas.*

It is of course tempting to speculate about the possible clinical uses of chalone.

During some of the meetings at this conference the problem of the role of chalones in carcinogenesis and in the treatment of cancer was discussed. All seem to agree that malignant tumors produce the chalone of their tissue of origin, and that the mitotic rate of tumors can be influenced by the specific chalone. However, tumors may be less sensitive than normal tissues to chalone inhibition of cell division. But, as regards the possible role of chalones in carcinogenesis and treatment of spontaneous cancers,

there was no agreement. Two views were expressed. One is the "optimistic" view, put forward mainly by Professor Bullough, stating that a disturbance in the chalone mechanism is probably a basic event in carcinogenesis and that treatment with chalones may be of great value in curing of cancer. The other view, more skeptical and also held by myself, states that there is no proof that a disturbance in the chalone mechanism is a basic event in carcinogenesis. It may well be that disturbances in the chalone mechanism are only secondary events. It seems to me much too early to make any statements about the possible role of chalones in the treatment of cancer. The two views sometimes clashed rather vigorously during the discussions, but since neither group had any convincing evidence that spontaneous cancers could or could not be treated favorably by chalones, the problem was left unsolved. Personally I feel that it is not sound reasoning first to accept that chalones are normal growth regulators, then to postulate that the malignant cells are less sensitive than normal cells to this growth regulator, and then to hope that chalones can be used directly for killing cancer cells. The mechanism of action of chalones may, of course, be more complicated than this. If chalones are also promoters of differentiation, the hope of using chalones in cancer treatment is more reasonable.

In personal discussions, especially with Dr. Houck, another possibility has been mentioned —that chalones may play a role as an adjuvant to cancer treatment. If cells are most sensitive to cytostatics and irradiation in DNA synthesis, in the G_2 or the M phase, it might be possible to use chalones to stop all cell divisions in a certain family of cells. If malignant cells are less responsive than normal cells to a given chalone concentration, it might be possible to regulate the dose and thus let the cancer cells go into cycle in a more or less synchronized manner, while the normal cells are still blocked by chalones in G_1. This would be the right moment for cytostatic treatment or irradiation.

I feel we have a long way to go before we can solve these problems. Patients should not be given unrealistic hopes by the too early publication of unfounded optimism.

Another possible clinical use is the treatment of psoriasis. If psoriasis is mainly characterized by disturbed differentiation and increased proliferation, the epidermal chalone may be of some use here if a chalone could be found that was stable enough to be used, for instance, in an ointment.

The most realistic prospects at present seem to lie in the use of the lymphocytic chalone to prolong graft survival. It seems generally accepted that cellular immunity is dependent upon cell division in a certain family of lymphocytes. If one family of lymphocytes alone can be kept nonproliferating by a specific chalone and if this chalone is a physiological substance with few harmful side effects and if it is species-unspecific, so that it can be produced for instance from cattle blood, this type of chalone use would be of great value for transplantation surgery.

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U.S. GOVERNMENT PRINTING OFFICE: 1973 O—491–609

DHEW Publication No. (NIH) 73-423
U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
Public Health Service
National Institutes of Health